Medical Library

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946 VOLUME 40, No. 4

THE BIOCHEMICAL JOURNAL

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THE UNIVERSITY OF CHICAGO PRESS
CANADA AND INDIA: MACMILLAN

Price 12s. 6d. net

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Studies on Diffusing Factors

1. THE KINETICS OF THE ACTION OF HYALURONIDASE FROM VARIOUS SOURCES UPON HYALURONIC ACID, WITH A NOTE UPON ANOMALIES ENCOUNTERED IN THE ESTIMATION OF *N*-ACETYL GLUCOSAMINE

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(*Received* 18 *February* 1946)

Hyaluronic acid has been shown to be made up of equal numbers of molecules of *N*-acetyl glucosamine and glucuronic acid (Meyer & Palmer, 1936), and to have a molecular weight of at least $2–5 \times 10^5$ (Blix & Snellman, 1945). The action of hyaluronidase upon it has been studied by following the loss of viscosity or of the power to form a clot with acetic acid in the presence of protein (Madinaveitia & Quibell, 1940; McClean & Hale, 1941; McClean, 1943; Robertson, Ropes & Bauer, 1940), and by estimating the liberated reducing groups (Meyer, Hobby, Chaffee & Dawson, 1940; Meyer, Chaffee, Hobby & Dawson, 1941). Meyer and his colleagues also estimated liberated *N*-acetyl glucosamine, but observed that the method which they used (Morgan & Elson, 1934) gave values far exceeding those to be expected when they studied the action of a pneumococcus enzyme on purified hyaluronic acid from human umbilical cords, and they did not pursue the matter further. They suggested that *N*-acetyl glucosamine liberated by the enzyme has 'a structure such as to give higher condensation and subsequently higher colour intensity than the synthetic compound'. Hahn (1945), in a paper which became available after the experiments described below had been completed, has shown that the breakdown products of hyaluronic acid which has been acted upon by mucopolysaccharases of *Cl. welchii* or testis (but not of leeches) are indeed more readily converted by dilute alkali than is *N*-acetyl glucosamine into compounds giving the characteristic purple colour with *p*-dimethylaminobenzaldehyde.

METHODS

As a preliminary step, a survey was made of the methods to be used for estimation of reducing sugar, glucosamine, and *N*-acetyl glucosamine under the experimental conditions to be described.

Reducing sugar. Somogyi's (1933) modification of the Shaffer-Hartman alkaline copper reagent has the advantage that it is unaffected by phenolic groups present in the protein and peptone impurities present in bacterial extracts. The reagent made up as described for estimation of glucose was tested with varying amounts of glucose, glucosamine hydrochloride, glucuronic acid and *N*-acetyl glucosamine. The values given by various batches of reagent were not quite constant, but in terms of glucose equivalence were approximately as follows: 1 mol. glucosamine HCl, 0·95 mol.; 1 mol. glucuronic acid, 0·43 mol.; 1 mol. *N*-acetyl glucosamine, 0·45 mol. glucose. It was observed that in the presence of citrate buffer the sensitivity of the reagent was depressed for all four of the compounds investigated, and control estimations were therefore always made with solutions of *N*-acetyl glucosamine and glucuronic acid in buffers corresponding to those used in the experiments.

It is probable that the low reducing equivalents found for glucuronic acid and *N*-acetyl glucosamine are due to lactone and oxazole formation respectively under the alkaline conditions employed in the estimation.

(Other methods of measuring reducing substances were tried. The Folin-Wu method for blood sugar, using copper tartrate and molybdic acid, gave 1 mol. *N*-acetyl glucosamine equivalent to 0·275 mol. glucose, and was abandoned as unsatisfactory for the purpose. The ferricyanide-ceric sulphate method of Miller & Van Slyke (1936) gave 1 mol. *N*-acetyl glucosamine equivalent to 0·91 mol. glucose. With this method, however, the reducing blank given by the enzyme and other protein was greatly increased.)

Glucosamine. This was estimated after acid hydrolysis either by the method of Palmer, Smyth & Meyer (1937) or of Sørensen (1938). The methods gave identical results.

N-acetyl glucosamine. The method of Morgan & Elson (1934) is satisfactory for the estimation of *N*-acetyl glucosamine in simple aqueous solution. Since the test conditions in this investigation involved various concentrations of buffer and of NaCl, the effect of these upon the estimation was studied. NaCl concentrations up to 2 M were without effect, but in the presence of M/6 citrate buffer, maximum colour development was obtained only after addition of considerably more 0·5 M-Na_2CO_3 solution than usual (0·33 ml. instead of 0·1 ml.), and after heating for 7 instead of 3 min. Even then the colour intensity was reduced, and standards were made up in buffer corresponding to the test conditions.

Viscosity reducing units. The viscosity reducing activity of the enzyme preparations was measured by the method of McClean & Hale (1941), in the presence of 0·1 M-NaCl and of M/6 citrate buffer at pH 4·6.

MATERIALS

Enzyme preparations

From ox testes. Several samples of enzyme were prepared, some from acetone-dried testicle powder from the Argentine, and some from a more recent acetone-dried

testicle powder kindly supplied by Glaxo Laboratories Ltd. One sample, prepared by the method of Morgan & McClean (1932), had an activity of 25 Viscosity Reducing Units (v.r.u.)/mg.; all others were prepared by the method of Madinaveitia (1941), and had activities of 110, 176, 210 and 460 v.r.u./mg. The preparations were freeze-dried in ampoules, which were opened fresh for each experiment, their activity being checked from time to time with the laboratory 'standard' hyaluronic acid against a dry, standard enzyme preparation.

From Cl. welchii. The organisms were grown in broth containing 0·5% crude hyaluronic acid, and the filtrate was used as the source of enzyme. The crude filtrates were stored under toluene at 4° and their activity (v.r.u./ml.) remained unchanged throughout the test period. One filtrate had an activity of 15 and the other 45 v.r.u./ml. A third preparation, partially purified by adsorption on alumina C_γ and subsequent elution with phosphate buffer, was also used. Its activity was 65 v.r.u./ml.

From streptococci. The organisms were grown in a broth similar to that used for *Cl. welchii*. One preparation consisted of the filtrate from a culture of a streptococcus group A (Lancefield) type 4 (Griffiths), and another from a culture of a streptococcus of group C, type 20. Their activity in terms of v.r.u./ml. was variable and unsatisfactory. Although at the time no explanation for this was available, later work by Hale (1944) has shown that hyaluronidase from streptococci is rapidly inactivated at pH 4·6, at which pH the v.r.u. assay was then made.

Hyaluronic acid preparations

Hyaluronic acid was prepared as a crude acetone precipitate from human umbilical cords (McClean & Hale, 1941), and this material was subjected to various procedures designed to remove impurities while avoiding any loss in viscosity.

(*a*) Solution in water at pH 9 followed by precipitation with a minimum volume (1–1¼ vol.) of ethanol saturated with potassium acetate.

(*b*) Grinding at room temperature with 90% (w/v) aqueous phenol. At this concentration, which was suggested by Dr W. T. J. Morgan, hyaluronic acid is insoluble, but most proteins and their derivatives go into solution.

(*c*) Shaking an already partly purified solution of hyaluronic acid with chloroform-amyl alcohol mixture, as originally described by Sevag (1934), in order to remove remaining protein as a chloroform gel.

A combination of methods (*a*) and (*b*) was used in most preparations, but they were incapable, even after many repetitions, of removing fine particles (resembling haematin) which imparted an opalescence. Potassium hyaluronate prepared in this way contained only 24–31% of glucosamine on dry-weight analysis, instead of the theoretical 41%. Method (*c*), repeated 3 or 4 times, was wasteful but yielded a clear, colourless, and highly viscous solution from which hyaluronic acid or its potassium salt could be precipitated by ethanol, and on analysis for nitrogen and glucosamine gave values in agreement with those of Meyer & Palmer (1936).

It is important to note that preparations of hyaluronic acid or its salts are markedly hygroscopic, and that unless careful precautions are taken samples are liable to contain 20% or more moisture.

Incubations

Except where otherwise stated, experiments were conducted at 37° in the presence of M/6 citrate buffer at pH 4·8, containing NaCl to 0·1 M. Incubation was carried out in stoppered tubes in presence of toluene. The substrate generally used was a preparation of hyaluronic acid purified to contain 70% of the potassium salt (as shown by glucosamine analysis), but a number of experiments were repeated with pure material. The concentration of substrate was 1·0 mg./ml., and the enzymes were used at a final concentration corresponding to 10 v.r.u./ml.

EXPERIMENTAL RESULTS

Comparison of hyaluronidases from various sources

Testis enzymes. All five preparations gave similar hydrolysis curves, of which a typical example is shown in Fig. 1. It can be seen from the curve (*a*) that when liberation of reducing substances and of

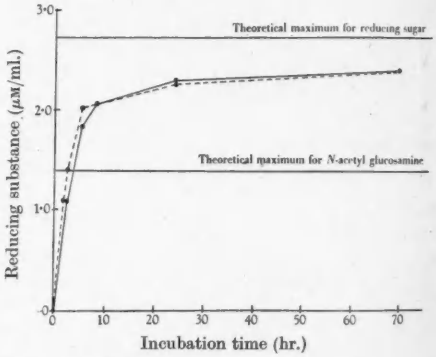

Fig. 1. Hydrolysis of hyaluronic acid by testis enzymes in the presence of M/6 citrate buffer pH 4·8 and 0·1 M-NaCl. - - - reducing substance calculated as glucuronic acid and *N*-acetylglucosamine; — apparent *N*-acetyl glucosamine.

N-acetyl glucosamine has ceased, the reduction equivalent (calculated on the assumption that reduction is due to an equimolar mixture of glucuronic acid and *N*-acetyl glucosamine) represents about 85% of the value calculated for complete hydrolysis, and (*b*) that the apparent amount of *N*-acetyl glucosamine liberated is 175% of that expected for complete hydrolysis. The ratio mol. apparent *N*-acetyl glucosamine liberated/mol. reducing substance (assuming an equimolar mixture of *N*-acetyl glucosamine and glucuronic acid) is approximately 1 : 1, whereas hydrolysis of hyaluronic acid to *N*-acetyl glucosamine and glucuronic acid requires a ratio of 1 : 2.

A small number of experiments were carried out in the presence of 0·1 M-NaCl with other buffer

solutions and with variation of the pH. Using M/36 citrate buffer at pH 4·8 or M/100 citrate-phosphate buffer at pH 4·8, the results were similar to those obtained under the standard conditions. With M/100 citrate-phosphate buffer at pH 6·0 the rate of hydrolysis was reduced by 25%, and with no buffer present at pH 7·0 it fell to one-tenth of the rate at pH 4·8. In every experiment, however, the same anomalous values for mol. apparent *N*-acetyl glucosamine liberated/mol. reducing substance were obtained, although at pH 6·0 and 7·0 the reducing substance (corrected) liberated was never more than 60% of theoretical.

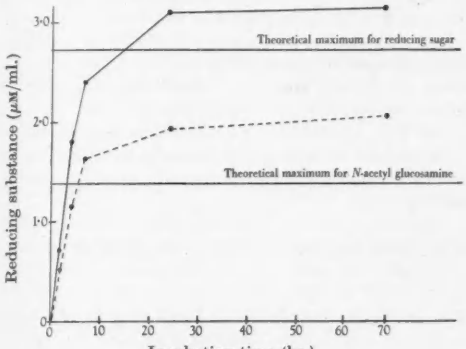
Theoretical maximum for reducing sugar
Theoretical maximum for N-acetyl glucosamine
Reducing substance (μM/ml.)
Incubation time (hr.)

Fig. 2. Hydrolysis of hyaluronic acid by *Cl. welchii* enzymes in the presence of M/6 citrate buffer pH 4·8 and of 0·1 M-NaCl. - - - reducing substance calculated as glucuronic acid and *N*-acetyl glucosamine. —apparent *N*-acetyl glucosamine.

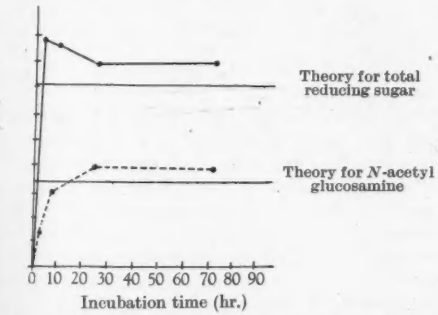

Fig. 3. Hydrolysis curve obtained with large amounts of crude *Cl. welchii* enzyme, showing liberation of maximum apparent *N*-acetyl glucosamine before maximum reducing substances. Legend as for Fig. 2.

Cl. welchii *enzymes*. The results of typical experiments are shown in Fig. 2. They differed from those obtained with testis enzymes in certain respects. Whereas the reduction equivalent (on the same assumption as above) at the end of hydrolysis reached an average value of 80% of theoretical for complete hydrolysis, the apparent *N*-acetyl glucosamine released was 250% of the theoretical amount. The ratio mol. apparent *N*-acetyl glucosamine/mol. reducing substance was consistently 1·7 : 1, using all three enzyme preparations.

Certain differences were also noted in the effects of changes of buffer and of pH. For a given buffer concentration, the rate of hydrolysis was the same at pH 7·0 as at 4·8, but hydrolysis was more rapid at buffer concentrations of M/100 or less than at M/25 or more. Occasionally, when large amounts of enzyme were used, the maximum value for *N*-acetyl glucosamine released was reached before the maximum for reducing substances, and the shape of the hydrolysis curve was as shown in Fig. 3.

Streptococcal enzymes. Only a few experiments were made with streptococcal enzymes, since at the time difficulty was experienced with the estimation of the strength of these enzymes in terms of v.r.u. (This was due, as has been shown by Hale (1944), to the instability of streptococcal enzymes under the experimental conditions used in the v.r.u. estimation.) Nevertheless, curves were obtained which bore out the findings with other enzymes. It was interesting to find that preparations made from the two different strains gave notably different results for the value of the ratio mol. *N*-acetyl glucosamine/mol. reducing substance liberated, namely, 1·3 : 1 for group A type 4 streptococcus preparations, and 2 : 1 for group C type 20 streptococcus. Typical curves are shown in Fig. 4.

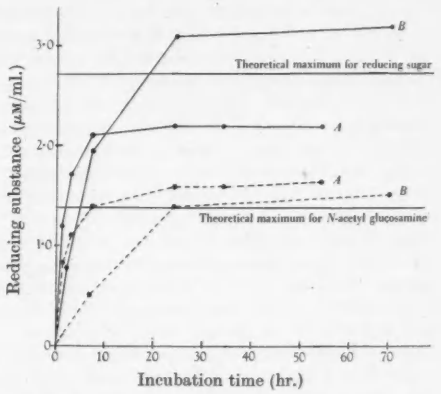

Fig. 4. Hydrolysis of hyaluronic acid by streptococcal enzymes in the presence of M/6 citrate buffer pH 4·8 and of 0·1 M-NaCl. (A) Group A, type 4. (B) Group C, type 20. - - - reducing substance calculated as glucuronic acid and *N*-acetyl glucosamine. — apparent *N*-acetyl glucosamine.

Comparison of the rate of hydrolysis with viscosity reduction

Sufficient data were accumulated to allow a comparison to be made between the rate of liberation of reducing substances and the rate of reduction of the viscosity of standard hyaluronic acid solutions by the various enzymes. The viscosity reducing activity (v.r.u.) was assayed by the method of McClean & Hale (1941), which requires the presence of M/6 citrate buffer at pH 4·8 and of 0·1 M-NaCl. In view of the observed variation of the rate of hydrolysis of hyaluronic acid by a given enzyme with the pH and salt concentration of the medium, and in the light of the observations of McClean (1943) upon the effect of these factors on other activities of hyaluronidase preparations, only those hydrolysis curves were chosen which satisfied the conditions prescribed for the v.r.u. estimation (McClean & Hale, 1941). The initial rate of hydrolysis was estimated graphically in terms of μM/ml./hr., using the values for reducing substances rather than the anomalous values for *N*-acetyl glucosamine.

For the four different preparations of testis enzyme, in eight experiments, the values for the ratio (rate of hydrolysis)/(v.r.u./ml.) lay between 0·024 and 0·036. Similarly, the two different preparations of *Cl. welchii* enzymes, in the three experiments only which were carried out under suitable conditions for comparison, gave values for this ratio between 0·022 and 0·032. No such value could be obtained for streptococcal enzymes owing to the failure of the v.r.u. assay mentioned above.

The estimation of the initial rate of hydrolysis from curves which were not planned with this purpose primarily in mind, and in which rather few points were obtained for the early parts of the curves (see figures), is a source of inaccuracy. Nevertheless, the results provide strong evidence in favour of the identity of the two actions of the preparations upon hyaluronic acid. This is particularly so in the case of the testis preparations, since their other activities such as β-glucosaminidase effect differed widely.

The anomalous values for N-acetyl glucosamine

The observation of Meyer *et al.* (1941) that the estimation of *N*-acetyl glucosamine under the usual conditions yields values higher than those expected has been amply confirmed by the foregoing investigations, although in none of them was the apparent excess of *N*-acetyl-glucosamine as great as in the experiments in which he investigated the action of pneumococcal hyaluronidase upon hyaluronic acid contained in a crude pleural exudate. It has been shown furthermore that enzymes from different sources give values which appear to depend upon the source from which the enzyme is prepared. The following observations were made concerning the anomalous values:

(1) Hydrolysis with acid following enzymic hydrolysis, and subsequent estimation of glucosamine showed that no new glucosamine molecules had been formed during incubation with the enzyme.

(2) Apparently high values for *N*-acetyl glucosamine were found only when hyaluronic acid was incubated with hyaluronidase. They were not found when hyaluronic acid was incubated alone, nor in the presence of other proteins, nor when enzyme was incubated with an equimolar mixture of glucuronic acid and *N*-acetyl glucosamine.

(3) The unexpected ratio of apparent *N*-acetyl glucosamine to reducing substances liberated by a given enzyme was independent of the purity of the substrate.

(4) This ratio was not affected by variations of the pH at which hydrolysis took place within the range 4·8–7·0, nor by pre-treatment of the hyaluronic acid with ascorbic acid and copper, although the viscosity was thereby reduced to that of water.

(5) The final ratio was found to be independent of the order in which the enzymes were added, when hyaluronic acid was treated for 48 hr. with testis enzyme and then for 48 hr. with *Cl. welchii* enzyme.

(6) When hydrolysis was carried to completion with testis enzyme at pH 4·8 inside a dialysis bag, the final values for reducing substances in the dialysate and in the diffusate were as 1·2 : 1 and for apparent *N*-acetyl glucosamine as 1·4 : 1. The substance giving the high values for *N*-acetyl glucosamine was therefore largely, if not wholly, diffusible under these conditions.

It was observed during the course of this investigation that when hyaluronic acid was incubated with enzyme at a pH between 6·0 and 7·0, treatment of the reaction mixture with standard Ehrlich's reagent (*p*-dimethylaminobenzaldehyde in acid solution) gave a purple colour directly, without the preliminary heating with alkali which is necessary in the ordinary estimation of *N*-acetyl glucosamine. The intensity of the colour obtained in this way increased as incubation proceeded, as is shown in Fig. 5, which illustrates the action of testis enzyme on hyaluronic acid at pH 7·0. In this figure the 'direct' colour has been compared with that produced by standard solutions of *N*-acetyl glucosamine treated with alkali in the usual way, and the theoretical value for true *N*-acetyl glucosamine which should be present is also included, having been calculated from the reducing values on the assumption that these are due to an equimolar mixture of glucuronic acid and *N*-acetyl glucosamine. It will be observed that even when the true values and those of the 'direct reacting substance' (DRS) are added together the combined total is still less than the apparent *N*-acetyl glucosamine.

DRS was not formed at pH 4·8, nor, in the absence of enzyme, was it formed when the products of hydrolysis by testis preparation at pH 4·8 were dialyzed and then brought to pH 7·0 by the addition of NaOH, followed by incubation at this pH for 24 hr. at 37°.

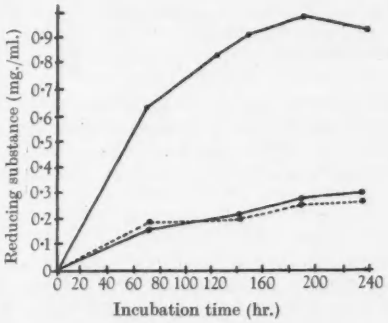

Fig. 5. Hydrolysis of hyaluronic acid by testis enzyme at pH 7·0 in the absence of buffer. The upper curve shows *N*-acetyl glucosamine as ordinarily estimated, and the lower shows the *N*-acetyl glucosamine equivalent of the substance reacting directly with Ehrlich's reagent. The dotted line shows the true *N*-acetyl glucosamine estimated from the reducing substances liberated.

In the hope that further investigation of the properties of DRS might shed light upon the anomalous values for *N*-acetyl glucosamine, and possibly also on the structure of hyaluronic acid, an attempt was made to isolate the end-products of enzymic hydrolysis carried out at pH 7·0 and at 4·8, and to study their composition. Although the work had to be stopped before any final conclusions could be reached, the following information was obtained:

A preparation of potassium hyaluronate (1 g.) containing 70% of the pure substance was dissolved in 25 ml. of 0·1% NaCl and hydrolyzed enzymically at pH 7·0, with no added buffer, until no further increase in reducing substances occurred. The enzyme used was 1 mg. of the most active testis preparation. At the end of this time the insoluble haematin-like impurities in the substrate were centrifuged down and discarded. They contained 5·1% N (dry weight) but no reducing substances and no glucosamine. The clear supernatant solution was evaporated to dryness under reduced pressure, and the residual semi-crystalline mass was used for experiment and analysis. A similar procedure was carried out with similar materials, but in the second experiment the pH was maintained at 4·8 by the addition of a few drops of dilute HCl. Finally, both procedures were repeated using 0·25 g. of the purest preparations of hyaluronic acid, and the results were confirmed qualitatively though not quantitatively since the digestion products and the DRS were required for spectroscopic examination.

Extraction of the hydrolysate with warm methanol (not anhydrous) removed almost completely the added chlorides, and any DRS which was present, while reducing substances and *N*-acetyl glucosamine were left behind substantially unchanged. This fact proved very convenient for examining the DRS and other end-products of hydrolysis separately.

Properties of the direct reacting substance. (1) DRS in aqueous or ethanolic solution when mixed with Ehrlich's reagent (as used in the estimation of *N*-acetyl glucosamine) develops in the course of a few minutes a deep purple colour, which gradually fades on standing for a few hours. The rates of colour development and of fading are accelerated by warming the solution. The shade of purple is the same as that which appears during the ordinary estimation of *N*-acetyl glucosamine.

(2) DRS is moderately soluble in methanol, ethanol and butanol, and in 90% aqueous acetone. It is not soluble in dry acetone, nor in cyclohexane. By extraction of the end-products of hydrolysis at pH 7 with butanol (which does not extract NaCl), and removal of solvent by distillation *in vacuo*, there were obtained about 25 mg. of a yellow hygroscopic sticky solid, which dissolved in water or ethanol to give a good purple colour with Ehrlich's reagent; this colour became very much more intense after standing overnight, and it is thought that a reversible polymerization of the DRS may have occurred during the heating of the butanol solution.

(3) It was found that DRS is volatile in moist butanol, but not in steam nor in acetone. (The experiments with butanol solutions were conducted by fractional distillation in a tall column, entrainment being unlikely.)

(4) The direct colour with Ehrlich's reagent is slowly discharged by addition of H_2O_2, and rapidly discharged by addition of $Na_2S_2O_4$. With HNO_2 it turns first to a rich yellow-brown colour and then fades. In all these respects the colour behaves in the same way as that which is formed during the ordinary estimation of *N*-acetyl glucosamine.

It is probable that a substituted oxazole is responsible for the colour in the reaction for *N*-acetyl glucosamine (Morgan & Elson, 1934; Morgan, 1938), being produced by ring formation during the period of heating with dilute alkali which is necessary before addition of the Ehrlich reagent. It is possible that the DRS is a substituted oxazole, which either pre-exists in hyaluronic acid, or is formed in small quantities during hydrolysis. Such a suggestion is supported by the observation that after *N*-acetyl glucosamine has been heated with dilute alkali the chromophore substance formed is extractable by *n*-butanol, and is volatile in *n*-butanol but not in steam, just as is the DRS.

Morgan (1938) also suggested that the chromophore substance in the *N*-acetyl glucosamine might be a substituted pyrazine formed by the condensation of two molecules of *N*-acetyl glucosamine. If

DRS were such a substance, pre-existing in hyaluronic acid, it should be readily detectable by characteristic absorption spectrum in the ultraviolet range. It was decided therefore to synthesize a pyrazine substituted in the 2:5 positions, which would presumably be substituted in a condensation product of *N*-acetyl glucosamine. 2:5-Dimethyl pyrazine was prepared from *iso*-nitrosoacetone, by the method of Gabriel & Pinkus (1893). The $HgCl_2$ compound so obtained was heated with KOH pellets, and the free dimethyl pyrazine was distilled off as a colourless oil (m.p. 11°) with a characteristic peppery smell. It was readily volatile in steam, and in small amounts gave a deep purple-pink colour with Ehrlich's reagent. The hue of this colour was not the same as that given by the DRS, and it did not fade either on standing at room temperature or after the addition of $Na_2S_2O_4$. In its behaviour towards H_2O_2 and HNO_2, however, it resembled the DRS.

The ultra-violet absorption spectra of 2:5-dimethyl pyrazine, of pure potassium hyaluronate, and of an enzymic partial hydrolysate of hyaluronic acid were very kindly measured by Dr E. R. Holiday. Although the dimethyl pyrazine possessed a well-defined spectrum, there was no evidence of any compound of its type present to an extent of more than 0·1 % in any of the hyaluronic acid preparations. It is therefore very unlikely that the colour given by the DRS, or by *N*-acetyl glucosamine treated with alkali, in the presence of Ehrlich's reagent is due to a 2:5 substituted pyrazine. Since oxazoles have no characteristic ultraviolet absorption spectra their presence could not be detected by this method.

Analysis of partial hydrolysates. The residues which remained after extraction of the evaporated partial hydrolysates with methanol were analyzed for ash, nitrogen, glucosamine, apparent *N*-acetyl glucosamine, reducing substances, and uronic acid. The uronic acid was estimated by the method of Kapp (1940), which involves heating in strongly acid solution with α-naphthyl resorcinol—a procedure which in itself leads to hydrolysis of hyaluronic acid and estimates therefore the total uronic acid in the material, rather than that part which is initially free. However, by taking half hourly samples and by following the development of the coloured condensation product between the uronic acid and the α-naphthyl resorcinol until maximum colour was developed, with (*a*) free glucuronic acid, (*b*) unhydrolyzed hyaluronic acid, and (*c*) the partial hydrolysates, it was possible to obtain an approximate value for the free uronic acid originally present in the partial hydrolysates.

The results confirmed those obtained upon the unseparated enzyme-substrate mixtures, and added little information, save that the values found for uronic acid (calculated as glucuronic acid from Kapp's formula), are in agreement with the supposition that half of the reducing substance liberated is glucuronic acid. Table 1 shows two different analyses.

Table 1. *Analysis of partial hydrolysates*

	% of dry weight	
	pH 4·8	pH 7·0
Nitrogen	3·3	3·1
Glucosamine after acid hydrolysis	28·9	28·0
Apparent *N*-acetyl glucosamine	30·0	14·5
Reducing sugar (as mixture of glucuronic acid and *N*-acetyl glucosamine)	27·4	15·0
Free glucuronic acid	13·0	8·0
Ash	4·0	10·0

DISCUSSION

Although the majority of the experiments described above were carried out with only partially purified hyaluronic acid, sufficient of them were repeated with highly purified hyaluronic acid to make it clear that the variations and anomalies were not due to impurities in the substrate. The data suggest therefore that the enzyme systems prepared from testis, *Cl. welchii* filtrates, and from streptococcal filtrates, act in different ways upon hyaluronic acid, although all of them cause hydrolysis with liberation of reducing substances and of substances which can be estimated by Morgan & Elson's method for *N*-acetyl glucosamine. This in itself is not surprising, since on the one hand the enzyme preparations were certainly not pure (see succeeding paper), and on the other they show marked differences in the way in which they are affected by pH and salt concentration. Furthermore, using the Tiselius (1942) carbon adsorption technique, Hahn (1945) has shown without question that enzymes from leeches and *Cl. welchii* act differently. It is perhaps rather surprising that the testis preparations, which were of widely differing activity, and the *Cl. welchii* preparations, should have given results which were so consistent for the type of enzyme which was used—suggesting either that in each case a single enzyme was involved in bringing about hydrolysis or that, if more than one enzyme was involved, the co-operating enzymes behaved very similarly during the process of purification or during storage. The enzymes from these two sources, when assayed under identical conditions of salt concentration and pH, showed so close a relationship between their power to reduce the viscosity of hyaluronic acid and to bring about its hydrolysis to small molecules that it is very probable that these two actions are brought about by the same mechanism.

The unexpected values obtained for *N*-acetyl glucosamine during the process of enzymic hydro-

lysis have been investigated but not explained. The most puzzling finding is the difference in the degree of anomaly from one type of enzyme to another. A possible explanation is that native hyaluronic acid contains linkages other than glycoside linkages between glucuronic acid and *N*-acetyl glucosamine; that these other linkages are such that groupings pre-exist in the hyaluronic acid which are readily capable of giving colour with Ehrlich's reagent; and that enzyme preparations from various sources attack hyaluronic acid in different ways (analogous to the actions of different amylases upon starch) so that more or less of these hypothetical groupings are revealed, according to the chain length of the units split off. Such a hypothesis is supported by the observation that no enzyme preparations hydrolyze hyaluronic acid to completion, and that the degree of hydrolysis is different for enzymes from different sources (Meyer *et al.* 1941). It agrees in the main with the conclusions of Hahn (1945) who claims recently to have separated two distinct components by the action of *Cl. welchii* enzymes and both oligosaccharides and monosaccharides from the action of testis enzymes on hyaluronic acid. The observation that during hydrolysis at pH 7·0 a substance (DRS) is produced which gives a purple colour with Ehrlich's reagent without previous heating in alkaline solution, and that this substance is produced in greater quantities during the action of *Cl. welchii* than of similar amounts of testis enzymes, can also be fitted in. Although the nature of this substance was not elucidated, it is interesting to note that a similar direct purple colour with Ehrlich's reagent was obtained with native cartilage from sheep nasal septum. More work on the detailed structure of hyaluronic acid is required before an acceptable explanation is likely to be forthcoming, but the above observations suggest that pre-formed ring structures may exist in hyaluronic acid and similar substances, or that the products of enzymic hydrolysis are peculiarly liable to spontaneous ring formation.

SUMMARY

1. The hydrolysis of hyaluronic acid by enzymes from ox testis, from *Cl. welchii*, and from two strains of streptococcus has been studied by following the liberation of reducing substances and of *N*-acetyl glucosamine.

2. The apparent *N*-acetyl glucosamine liberated is in every case in excess of the theoretical amount, and the degree of excess depends upon the source from which the enzymes are derived.

3. During hydrolysis at pH 6·0 or 7·0 a substance is liberated which reacts with *p*-dimethylaminobenzaldehyde in acid solution (Ehrlich's reagent) to give a purple colour, without any pre-treatment with alkali.

4. This substance is not a 2:5 pyrazine derivative, nor is such a derivative likely to be concerned in the normal estimation of *N*-acetyl glucosamine.

5. It is suggested that hyaluronic acid contains, besides glycosidic linkages between glucuronic acid and *N*-acetyl glucosamine, pre-formed ring compounds involving *N*-acetyl glucosamine—or that the enzymic hydrolysis products are peculiarly liable to form such compounds.

6. Evidence is given for the identity of the hydrolyzing activity and of the viscosity reducing activity of enzymes from testis and *Cl. welchii*.

I wish to thank Dr D. McClean and Dr W. T. J. Morgan for help and advice during this investigation, and Dr H. J. Rogers for valuable suggestions in preparing the manuscript.

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Studies on Diffusing Factors

2. THE ACTION OF HYALURONIDASE PREPARATIONS FROM VARIOUS SOURCES UPON SOME SUBSTRATES OTHER THAN HYALURONIC ACID

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(*Received* 18 *February* 1946)

The action of enzyme preparations possessing hyaluronidase activity upon a number of substrates other than hyaluronic acid has been studied by Meyer and his colleagues (1940, 1941), by Madinaveitia & Stacey (1944), and by McClean & Hale (1941). The enzymes, for whose purity no claims were made, were prepared from testis, leech, pneumococcus, streptococcus, and *Cl. welchii*, and the substrates tested included a wide range of natural mucopolysaccharides and glucuronides, and two synthetic β-glucosaminides. These studies, which were mainly qualitative, showed that there was a wide variation in the number of substrates which were attacked, depending upon the source of enzyme. Although there is little doubt that in many of these instances enzymes distinct from hyaluronidase were involved, it has been questioned (East, Madinaveitia & Todd, 1941) whether β-glucosaminidase activity is essential to the complete breakdown of hyaluronic acid, and it seemed that this was a point worth resolving. In the course of repeating, and confirming, some of the experiments of Meyer and his colleagues it was observed that testis enzyme preparations hydrolyzed chondroitin sulphuric acid almost as rapidly as hyaluronic acid, and the kinetics of this action were briefly studied also in the hope that the enzyme might prove useful in studying the detailed structure of chondroitin sulphuric acid. This particular action of testicular hyaluronidase preparations has been observed independently by Madinaveitia & Stacey (1944), who recorded, however, only the effect on the viscosity of the substrates which they tested.

METHODS AND MATERIALS

The methods used have already been described in the previous paper, except for that used for the estimation of phenol.

Estimation of phenol in the presence of bacterial culture media. Phenol can readily be estimated colorimetrically with the reagent of Folin and Ciocalteu, but when the enzyme preparations are contaminated with peptone the phenolic groups in the latter are also estimated and the blank values are hopelessly large. It was found, however, that phenol can be quantitatively separated from bacterial culture broth by steam distillation for 5 min. in the modified micro-Kjeldahl apparatus described by Markham (1942). Broth or bacterial filtrates alone when distilled in this way at pH 5·0 yielded no volatile phenols, whereas when 0·05–0·5 mg. of phenol was added at the same pH, 95% of the added phenol was found in the distillate and could be estimated in the usual way. This fact made it possible to follow the hydrolysis of β-phenyl *N*-acetyl glucosaminide by estimation of liberated phenol.

Enzymes. In addition to the preparations described in the previous paper β-glucosaminidase was prepared from the intestines of garden snails and of water snails by extraction with glycerol as described by Neuberger & Pitt-Rivers (1939).

Substrates. β-Phenyl *N*-acetyl glucosaminide was prepared according to Neuberger & Pitt-Rivers (1939). Cartilage was prepared from the nasal septum of a young sheep, freed from adherent material, and dried. It contained approximately 10% chondrosamine after hydrolysis with acid.

RESULTS

(1) *The action upon β-glucosaminides*

Approximately 50 viscosity reducing units of the enzyme preparations were incubated at 37° with 10 mg. of the substrate in the presence of M/6 citrate buffer pH 4·8 and of 0·1 M-NaCl (total vol. 10 ml.). Samples were withdrawn at intervals for estimation of liberated reducing sugars, *N*-acetyl glucosamine, and of phenol when present.

A typical curve showing hydrolysis of β-phenyl and of β-methyl *N*-acetyl glucosaminides by a relatively crude testis enzyme (25 v.r.u./mg.) is given in Fig. 1. It will be noted that the aromatic glucosaminide is hydrolyzed faster than the aliphatic, in keeping with the observations of Neuberger & Pitt-Rivers on other glucosaminidases. The amounts of liberated reducing substance (corrected as *N*-acetyl glucosamine), of free *N*-acetyl glucosamine, and of free phenol corresponded very closely, and there was no apparent excess of *N*-acetyl glucosamine such as is found when hyaluronic acid is hydrolyzed by the same preparations (Humphrey, 1946).

No correlation was found between the β-glucosaminidase activity of the preparations and their hyaluronidase activity, as measured by release of

reducing groups from hyaluronic acid or by viscosity reduction. If the degree of hydrolysis of 10 mg. of β-phenyl *N*-acetyl glucosaminide after 24 hr. incubation at 37° is taken as a measure of the β-glucosaminidase activity, the relative activities of approximately 50 v.r.u. of different hyaluronidase preparations were found to be: testes preparations (*a*), (*b*), (*c*), (*d*), 100, 6, 16, $<4\%$ hydrolysis respectively; *Cl. welchii* preparations (*a*) and (*b*), 45 and 3% respectively; streptococcus A, 4 and streptococcus C, 20, negligible hydrolysis.

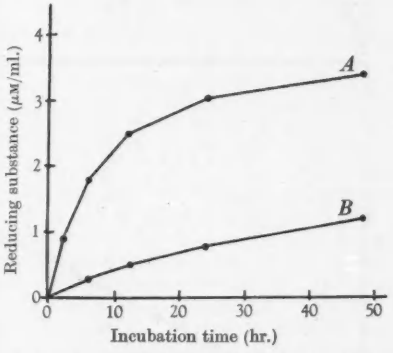

Fig. 1. Hydrolysis of β-phenyl (*A*) and β-methyl *N*-acetyl glucosaminide (*B*) by testis preparation at pH 4·8.

Furthermore, although the snail intestinal preparations contained powerful β-glucosaminidase, neither of them hydrolyzed hyaluronic acid or showed diffusion following intracutaneous injection in rabbits.

(2) *The action upon chondroitin sulphuric acid and cartilage*

The enzymes used were two testis preparations, one with an activity of 25 v.r.u./mg. and the other 460 v.r.u./mg.; one crude *Cl. welchii* filtrate, and a crude filtrate from a culture of streptococcus group C type 20. Experiments were carried out in the presence of M/80 citrate-phosphate buffer at pH 4·8 and 6·0, at an enzyme concentration of approximately 5 v.r.u./ml. and a substrate concentration of 2·5 mg. chondroitin sulphuric acid/ml. or 12·5 mg. cartilage/ml. The mixtures were incubated under toluene at 37°, and samples were withdrawn at intervals for estimation of reducing substances and *N*-acetyl glucosamine.

The following results were obtained:

(*a*) The streptococcal enzyme was inactive.

(*b*) The *Cl. welchii* preparation caused a very slow liberation of reducing substance and of a substance giving colour in the *N*-acetyl glucosamine estimation, but the rate was not comparable with the rate of hydrolysis of hyaluronic acid by the same preparation. These results agree with the observations of Meyer *et al.* (1941).

(*c*) The testis enzymes, despite marked difference in purity, behaved in all respects similarly. Fig. 2 shows the action of one of the preparations upon chondroitin sulphuric acid and upon hyaluronic acid under the same conditions at pH 4·8 and 6·0. The rate of liberation of reducing substances is almost the same from either substrate, and the effect of pH is the same. When chondroitin sulphuric acid is hydrolyzed, however, the apparent *N*-acetyl glucosamine does not show the excessive values found when hyaluronic acid is hydrolyzed. Since the products of hydrolysis of chondroitin sulphuric acid were not investigated further it is not possible to state what the 'theoretical' values for reducing substances and apparent *N*-acetyl glucosamine should be. *N*-acetyl chondrosamine should behave in the same way as *N*-acetyl glucosamine, but it is probable that unless a sulphurase is also present, the hydrolysis products are rather glucuronic acid and a sulphuric acid ester of *N*-acetyl chondrosamine, and the latter would require further investigation.

When finely ground cartilage was the substrate, reducing substances and a substance reacting as *N*-acetyl glucosamine were liberated at a rate approximately half that when chondroitin sulphuric acid was the substrate, whereas cartilage incubated without enzyme was unaffected. It is likely that the insolubility of cartilage was a limiting factor.

DISCUSSION

Since testis enzyme with almost negligible β-glucosaminidase activity and streptococcus enzymes with negligible β-glucosaminidase activity can hydrolyze hyaluronic acid in characteristic fashion, it is clear that such activity is not necessary to hyaluronidase, and this is borne out by the failure of β-glucosaminidase preparations to show hyaluronidase effects. The powerful action of testis enzyme on cartilage and on chondroitin sulphuric acid is interesting, and the parallel behaviour of activity against these substrates and against hyaluronic acid suggests that the same enzyme may be involved for both. The substrate specificity among sulphuric acid-containing mucopolysaccharides is also interesting, for, in addition to chondroitin sulphuric acid, synthetic trisulphuric acid ester of hyaluronic acid and the sulphur-containing pig-skin polysaccharide are hydrolyzed (Meyer *et al.* 1940), whereas heparin and mucoitin sulphuric acid are not.

SUMMARY

1. β-Glucosaminidase activity of a number of preparations of hyaluronidase has been tested.

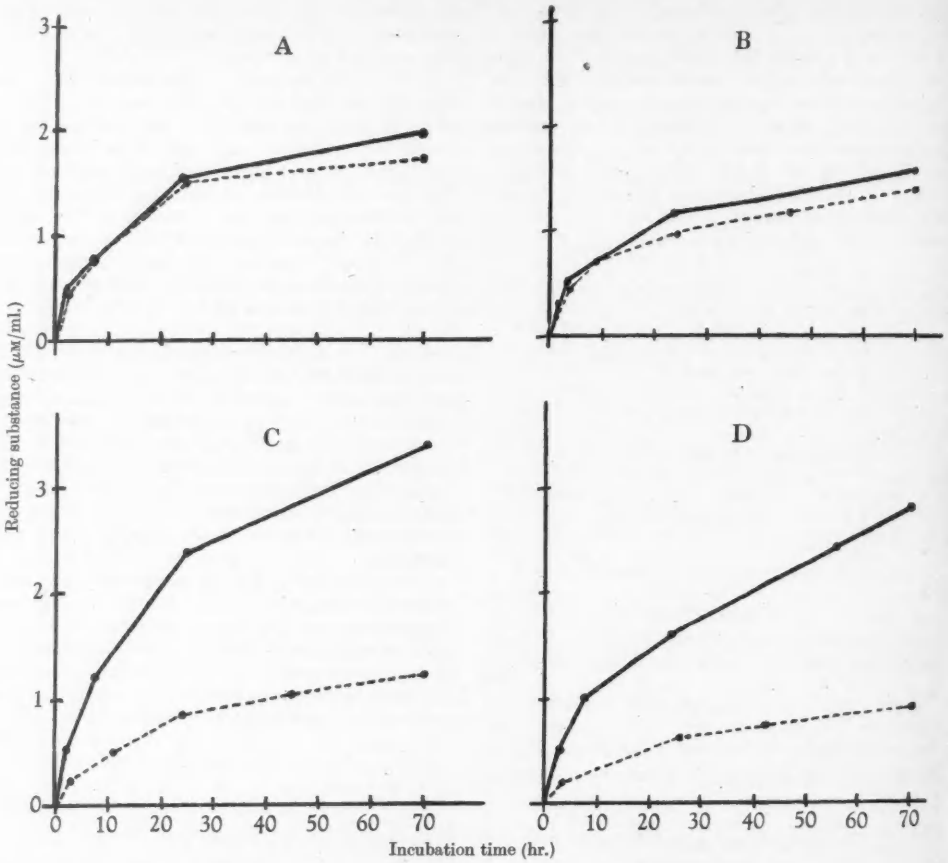

Fig. 2. Hydrolysis of hyaluronic acid and of chondroitin sulphuric acid under similar conditions by testis preparation. (A) Liberation of reducing substances at pH 4·8. (B) Liberation of reducing substances at pH 6·0. (C) Liberation of apparent *N*-acetyl amino-sugar at pH 4·8. (D) Liberation of apparent *N*-acetyl amino-sugar at pH 6·0. The values for reducing substances are expressed as mol. glucose, and have not been corrected. To convert to mol. reducing sugar, assuming an equimolar mixture of glucuronic acid and *N*-acetyl glucosamine, the values shown must be multiplied by 2·15. —— Hyaluronic acid. --- Chondroitin sulphuric acid.

There is no correlation between the β-glucosaminidase content and the ability to hydrolyze hyaluronic acid.

2. Two preparations of testis hyaluronidase acted upon chondroitin sulphuric acid to release reducing groups at a rate equal to that of hyaluronic acid under similar conditions. In the case of chondroitin sulphuric acid, estimation of liberated substance giving the reaction of *N*-acetyl glucosamine (or chondrosamine) does not show the apparent excess found in the case of hyaluronic acid. Native cartilage was hydrolyzed at a rate about half that of chondroitin sulphuric acid.

I wish to thank Dr C. R. Harington for a gift of β-methyl *N*-acetyl glucosaminide, and Dr W. T. J. Morgan for a gift of a sample of Ca-Mg salt of chondroitin sulphuric acid originally prepared by Dr E. Jorpes.

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The Polarographic Estimation of Steroid Hormones

I. POLAROGRAPHY OF NEUTRAL 17-KETOSTEROIDS IN URINARY EXTRACTS

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(*Received* 25 *February* 1946)

The need for the development of dependable methods for the determination of the steroid hormones and their metabolites in biological material, and the value of such determinations for clinical endocrinology, requires no emphasis. The present paper deals with the determination of neutral 17-ketosteroids in urine. This group includes androsterone, *iso*dehydroandrosterone, *iso*androsterone and $\Delta^{3,5}$ androstadien-17-one as well as other androstane derivatives present only in small amounts. In women, the neutral 17-ketosteroid output may be taken as a measure of adrenal-cortical function, while in men it includes metabolites of both testicular and adrenal-cortical hormones. The neutral 17-ketosteroid output has proved of considerable clinical value in the diagnosis of adrenal tumours. In the past such estimations have been carried out by rather unspecific colour reactions and the required specificity gained by exhaustive fractionation of the extracts. Even so, the wide variation in the properties of the extracts makes it very difficult to devise a relatively simple process suitable for all cases. This is well exemplified by the varied factors suggested for the correction of the Zimmermann 17-ketosteroid colour for the intrinsic colour of the extract.

In 1940, Wolfe, Hershberg & Fieser suggested a new approach to the problem. They showed that certain ketonic steroids condensed with the Girard T reagent (tetramethylammonium acethydrazide chloride) to give water-soluble hydrazones, which under suitable conditions yielded well-defined polarographic waves (Fig. 1*a*). The wave span was proportional to the steroid concentration over a considerable range, and amounts of the order of 10 μg. could be estimated with accuracy. This characteristic wave with a half-wave potential at about $-1{\cdot}4$ V. appears to be given only by substances in which the ketone group is attached to a saturated five-membered ring. This wave would therefore appear to be specific for 17-ketosteroids. An α-β unsaturated ketone in a six-membered ring also shows a wave under these conditions, but at a lower half-wave potential of $-1{\cdot}1$ V. Wolfe *et al.* (1940) claimed that the two types of ketonic steroid could be determined simultaneously in the same specimen of urine. Later they (Hershberg, Wolfe & Fieser, 1941) extended the method to hydroxysteroids by effecting Oppenauer oxidation to the corresponding ketone with polarography of the Girard T hydrazone. They also applied the method to the determination of neutral 17-ketosteroids in urine and obtained results substantially the same as those given by the Zimmermann colour reaction. The method obviously could be extended to the oestrogens, progesterone and the cortical steroids, and the extremely high sensitivity of the polarographic technique makes it a promising micro-method of high specificity.

In repeating this work we have found that while pure 17-ketosteroids give a well-defined wave, certain urinary extracts of the neutral 17-ketosteroids contain material which distorts the wave form in two respects. In the first place the final discharge occurs at a lower voltage, so that the point of inflexion of the wave is difficult to distinguish (Fig. 2*a*). In the second place a second small wave extending from about $-1{\cdot}0$ to $-1{\cdot}3$ V. makes it difficult to establish a true base line (Fig. 2*a*). The interfering material is ketonic in nature since extracts not treated with Girard T reagent give no polarographic wave. The position of the interference suggests that it may be due to 3- and 20-ketosteroids present in the extract. We have attempted to overcome these difficulties in two ways: (*a*) by decreasing the rate of rise of applied

voltage, as suggested by Werthessen & Baker (1945). While the spectacular improvement claimed by these workers was not obtained, nevertheless, as can be seen from Fig. 1*b*, *c*, and Fig. 2*a*, *c*, a considerable advantage does accrue from the use of this technique; (*b*) by destruction of a considerable proportion of interfering material by oxidation of the extract with potassium permanganate in aqueous dioxan (Fig. 2*d*) under conditions which do not affect 17-ketosteroids.

This improved technique has been applied to a study of the neutral 17-ketosteroid excretion over 24 hr. in 24 healthy young men (medical students) in order to establish the normal average ketosteroid output, and its range of variation, by the polarographic method.

EXPERIMENTAL

Apparatus

The greater part of the work described was carried out with a Tinsley ink recorder polarograph, while in some of the earlier experiments a Cambridge photographic instrument was used. The sensitivities of the Tinsley instrument used in this work were as follows:

40 μa. = 100 scale divisions = 89 mm. (sensitivity A)
20 μa. = 100 scale divisions = 89 mm. (sensitivity B)
10 μa. = 100 scale divisions = 89 mm. (sensitivity C)
5 μa. = 100 scale divisions = 89 mm. (sensitivity D)

The polarograph potentiometer was calibrated against an external potentiometer and Weston cell. Variations in applied potential from the indicated value did not exceed 0·2%. In those experiments where the rate of rise of applied potential was decreased in order to give a greater wave-spread, a 500 ohm wire-wound variable resistance was inserted in one battery lead, and by adjustment of this and the internal standardizing resistance the applied potential was set to 0·60 times the indicated value. The dropping electrode was of the constant head type described by Mueller (1940). A typical capillary used had the values: drop time $t = 1{\cdot}84$ sec.; average drop weight = 6·90 mg.; capillary constant $k = m^{\frac{2}{3}}\, t^{\frac{1}{6}} = 2{\cdot}68$ (m = mass of mercury dropping per sec.).

A series of experiments with different values of t showed that the range 2·0–2·5 sec. gave the best wave definition. The cell assembly used was very similar to that described by Wolfe *et al.* (1940). The use of a saturated calomel electrode instead of the mercury pool did not influence the wave form. Later experiments were carried out at 25° ± 0·2°.

Experiments designed to improve the extraction technique

Four parallel experiments were carried out, two using benzene as the extraction solvent, and two using carbon tetrachloride. Emulsions were somewhat worse with the latter; identical values for the wave height were obtained in all four cases; benzene has therefore been used throughout.

No advantage was secured by extracting with 100 ml. portions of benzene rather than with 50 ml. volumes. With 25 ml. volumes, however, troublesome emulsions formed; the extraction was therefore made with four portions each of 50 ml. of redistilled benzene.

Saturation of the urine after hydrolysis with sodium chloride appeared to lead to less highly coloured benzene extracts and to decrease the tendency for emulsification. Hence after hydrolysis, 26 g. of sodium chloride was added per 100 ml. of urine.

Experiments designed to improve the polarograms

(*a*) *Purification of the extract.* Attempts to purify the extract still further were at first directed towards removing 3- and 20-ketosteroids. Accordingly, an attempt was made to apply the iodoform reaction on a micro-scale to the final extract. However, while addition of iodine in alkaline solution to control samples of progesterone did remove the wave almost completely, the same treatment was found to reduce the *iso*dehydroandrosterone wave to a small fraction of its original height; tribromomethane had a similar effect. The use of potassium permanganate in aqueous dioxan (1 : 1) was then investigated. It was found that with 0·005 N-permanganate, the deoxycorticosterone-acetate wave could be reduced to one-quarter of its original height after treatment for 1½ hr. at room temperature; by raising the permanganate concentration (0·1 ml. 0·1 N-permanganate in 0·8 ml. aqueous dioxan), the wave was reduced almost to zero after 15 min. treatment. The polarogram of cholesterone was likewise largely eliminated by this treatment. *iso*Dehydroandrosterone under these conditions showed no diminution in wave span, as shown in Table 1.

Table 1. *Recovery of* iso*dehydroandrosterone* (I.D.A.) *added to urinary extracts before oxidation*

I.D.A. present in extract (μg.)	I.D.A. added (μg.)	I.D.A. present after oxidation (μg.)	I.D.A. recovered (%)
72·0	50	120·4	96·8
56·5	50	106·5	100·0
53·0	50	103·0	100·0
		Mean	98·93

(*b*) *Base solution.* Various ions have been studied as components of the base solution. Lithium, potassium and sodium ions showed no difference at the same pH. If ammonium chloride is used, it tends to lose ammonia after admixture with sodium hydroxide and is therefore unsuitable. The composition of the base solution which has finally been adopted in this work is: 0·5 N-sodium chloride (1 vol.), 0·2 N-sodium hydroxide (2 vol.) and water (1 vol.). This mixture has been found satisfactory, since the components may be mixed in bulk and stored for a considerable period without deterioration; this avoids the procedure used by Wolfe *et al.* (1940) of adding the components separately for each estimation.

(*c*) *Voltage spread.* The improvement suggested by Werthessen & Baker (1945) has been tried out and, as shown in Figs. 1*b*, *c*, and 2*a*, *c*, considerable advantage has been gained. Essentially the variation consists of applying a narrower range of potential to the system over the same period of time, i.e. while the potentiometer records a change of voltage of from 0 to −3 V., the instrument is so adjusted (vide under Apparatus) that the actual change is only 0·6 of that recorded, viz. from 0 to −1·8 V., the potentiometer

drum rotating at the same speed as in the original recordings. Hence the net effect is a 'spread' of the wave. It is seen that where the curve of the crude extract is difficult to read, the application of the 'spread' potential method facilitates the reading of the point of inflexion.

Estimation of neutral 17-ketosteroids in urinary extracts

Reagents

Acetic acid. This was prepared as described by Wolfe *et al.* (1940). Acetic anhydride (A.R.) was carefully fractionated and the pure product was refluxed for 1 hr. with glass-distilled water (rather less than 1 equiv.); the acetic acid was then fractionated from the small amount of excess acetic anhydride.

Girard reagent T. The commercial reagent was recrystallized five times from ethanol; no appreciable difference was found, however, between the polarographic waves given with the recrystallized and the commercial products. Contrary to the findings of Wolfe *et al.* (1940) we have not found that the reagent has necessarily deteriorated when it smells of trimethylamine. The solution of the reagent in acetic acid was always made up freshly each day, by dissolving 20 mg. of the reagent in 0·2 ml. of pure acetic acid.

Base solution. Sodium chloride (A.R.), sodium hydroxide (A.R.) and glass-distilled water were used throughout.

Mercury. All mercury was cleaned in the usual way with dilute nitric acid followed by thorough washing with distilled water.

Benzene. Benzene was redistilled before use. Benzene recovered from the extractions by distillation was found to be satisfactory for polarographic use without further purification.

Sodium carbonate solution. A 9% solution of sodium carbonate (A.R.) was used for washing the extract.

Sodium hydroxide. A 2 N-solution of sodium hydroxide (A.R.) in recently boiled glass distilled water was prepared and stored in clear glass bottles (not waxed).

Sodium hyposulphite. A 10% solution of fresh, powdered sodium hyposulphite in 2 N-sodium hydroxide was prepared freshly for each estimation and kept well stoppered.

Procedure.

A 24 hr. specimen of urine is collected in a bottle containing 7 ml. conc. HCl and examined within 24 hr. of collection. A portion is taken which may be expected to contain between 0·5 and 1 mg. of ketosteroids (usually about 1/15 vol. was used) and heated rapidly to boiling. For each 100 ml. 15 ml. conc. HCl are added and the boiling continued for exactly 10 min. under reflux. The liquid is cooled immediately, treated with 26 g. of NaCl and extracted 4 times each with 50 ml. of redistilled benzene. These benzene extracts are combined and washed twice with a 9% solution of Na_2CO_3, which breaks down any residual emulsion and takes out most of the brown colour. The combined extracts are then evaporated under reduced pressure until the volume is rather less than 100 ml., transferred to a 125 ml. separating funnel and the flask washed out with sufficient benzene to bring the total volume to 100 ml. Phenols are removed by four washings each of 25 ml. of 2N-NaOH; the pale pinkish-brown liquid is then shaken with 25 ml. of a freshly prepared 10% solution of sodium hyposulphite in 2N-NaOH, for 3 min. (or until the colour changes to yellow), keeping the separator stoppered during the whole time (Wolfe *et al.* 1940). Finally the extract is washed four times each with 25 ml. of water and allowed to stand until all droplets of water have settled out. The practically colourless benzene extract is distilled to dryness *in vacuo* in a bath at about 60°, final evaporation being done at room temperature. The residue is dissolved in 1·0 ml. of redistilled benzene and kept in a tightly stoppered bottle. This forms the *crude extract.* The polarographic estimation is carried out as soon as possible.

Further purification of the crude extract is carried out as follows: 0·1 ml. of 'crude extract' is evaporated to dryness in a small tube *in vacuo.* The residue is dissolved in 0·5 ml. of freshly redistilled dioxan, and 0·1 ml. 0·1 N-$KMnO_4$ and 0·25 ml. of 0·01 N-periodic acid are added and well mixed by gentle shaking. The tube is stoppered and left to stand at room temperature for 15 min. Excess $KMnO_4$ should be present; a further 0·1 ml. may be added if required. It is then transferred quantitatively to a small glass-stoppered separating funnel, washing in with 5 ml. of benzene and 5 ml. of water. The solution is extracted with benzene and the lower layer discarded. Excess of permanganate is removed by shaking with 3 ml. of a freshly prepared 10% solution of sodium hyposulphite in 2N-NaOH. After washing four times with water the benzene extract is allowed to clear and is evaporated to dryness in a small tube; this is the *oxidized extract.*

For preparation of Girard T hydrazones 0·1 ml. of the 'crude extract' is evaporated to dryness *in vacuo* at 30° in a small tube, while the 'oxidized extract', representing 0·1 ml. of the crude extract, is used directly; to the residue in either case is added 0·02 ml. of a freshly prepared solution containing 20 mg. of Girard T reagent in 0·2 ml. of pure acetic acid. The liquid is carefully rotated in the tube so as to cover the extract residue; it is then stoppered and heated in a boiling water bath for 2 min. The cooled product is treated with 2 ml. of the base solution (see Reagents), and shaken to dissolve it. The solution should be clear or only slightly cloudy at this point; in our experience, the polarogram is interfered with if solid particles remain suspended in the liquid, these appearing to cause irregularities in the curve. The clear solution is transferred to the polarograph cell, about 1 ml. of mercury is added, and the liquid deoxygenated by passing a fairly rapid stream of nitrogen for 3 min. The dropping electrode is then inserted and the solution is subjected to an increasing potential,

beginning at −0·9 V. and finishing at −1·8 V. Sensitivity 'B' is usually chosen for a solution of unknown ketosteroid content; other sensitivities may then be used according to the wave-span shown on this first curve.

RESULTS

Before examining the results which have been obtained from the estimation of neutral 17-ketosteroids in urinary extracts, it is necessary to discuss the method used to evaluate the polarograms obtained. In each case studied, two polarograms were made, one on the 'crude extract' (Fig. 2*a*) and one on the 'oxidized extract' (Fig. 2*d*). The wave span was in each case the height of the perpendicular drawn from the point of inflexion of the wave to the base line. (This is seen most clearly in Fig. 1*a*.) Table 2 shows the figures obtained for the 'crude' and 'oxidized' extracts. From Fig. 2*d* it will be seen that although the initial wave between −1·0 and −1·3 V. has been reduced, it has not been entirely eliminated. In order to correct the height of the wave for this interfering substance, an evaluation has been adopted whereby a line is drawn parallel to the base line through the point at which the 'real' wave due to 17-ketosteroids begins. The intercept of this line on the perpendicular gives the value to be deducted from the total wave span, as illustrated in Fig. 2*e*. These values are termed the 'corrected values', as tabulated in the last column, Table 2. The standard wave-span for 100 μg. of *iso*dehydroandrosterone has been taken as 20 mm. at sensitivity C, all experimental wave-spans being related to this standard. The average value of the 'corrected' figures is 15·7 mg./24 hr., with a standard deviation of 2·47 mg./24 hr., and a coefficient of variation of 15·7 %. Hence the probable range for 95·6 % of all results (mean ± 2σ) is: 10·8–20·6 mg. for normal young males.

Table 2. *Urinary excretion of neutral 17-ketosteroids (mg./24 hr.) in 24 normal young men*

Experimental no. index	Urine volume (ml./24 hr.)	Neutral 17-ketosteroids (mg.) estimated for 'Crude' extract	'Oxidized' extract	'Corrected' value
M 3	1820	24·1	18·2	15·6
M 4	990	28·8	14·8	10·9
M 5	1480	32·2	17·0	14·7
M 6	675	27·6	20·4	16·5
M 7	1340	—	19·8	16·5
M 8	1350	18·9	14·6	13·5
M 9	2580	25·3	21·8	16·8
M10	1670	31·1	22·1	17·2
M11	1450	21·0	18·1	14·9
M12	845	36·3	23·0	20·2
M13	1085	24·4	14·0	12·4
M14	1960	—	—	14·7
M16	1510	23·0	18·9	15·7
M17	1665	29·6	21·6	16·3
M18	1740	24·5	17·4	13·5
M19	2460	33·3	19·1	14·2
M20	1910	35·8	25·7	21·0
M21	900	27·3	21·4	16·6
M22	1030	30·1	17·5	13·7
M23	1500	36·0	25·1	19·5
M24	1570	29·8	24·9	18·5
M25	2070	28·7	21·3	17·6
M26	2090	30·2	20·4	15·1
M27	1700	23·0	17·8	12·8
Mean value		28·2	19·8	15·7
Standard deviation		4·8	3·25	2·48
Coefficient of variation (%)		17·2	16·4	15·8
Range (Mean ± 2σ, for 96 % results)		18·6–37·8	13·3–26·3	10·8–20·6

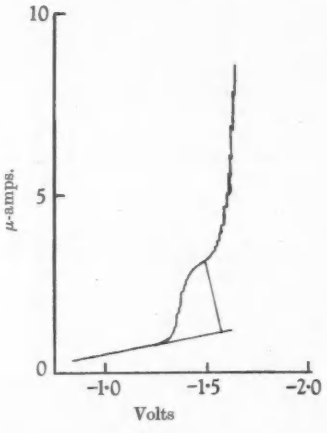

Fig. 1*a*.

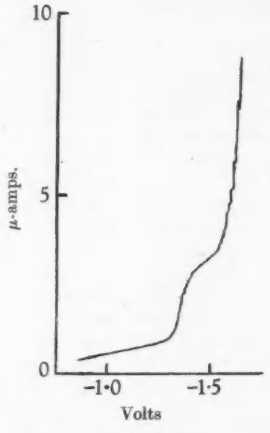

Fig. 1*b*.

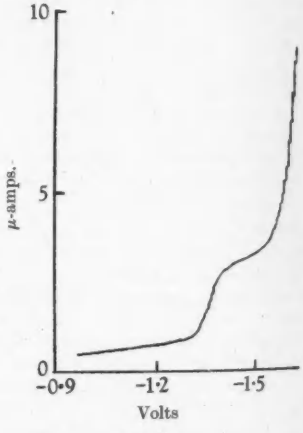

Fig. 1*c*.

Fig. 1. Polarograms of *iso*dehydroandrosterone. Sensitivity C. *a*, 100 μg. *iso*dehydroandrosterone + Girard reagent T. *b*, *c*, 100 μg. *iso*dehydroandrosterone + Girard reagent T, showing unspread wave (*b*) and spread wave (*c*).

A further series of estimations has been undertaken, with a view to comparing the polarographic and colorimetric methods of assay on normal men and women. These results together with a fuller discussion of the above figures in relation to relevant published data, will be published later.

potential effects a 'spreading' of the polarographic wave, has been found to facilitate considerably the assessment of the point of inflexion.

3. Oxidation of the urinary extract with permanganate before polarography has been shown to remove much of the interfering material which

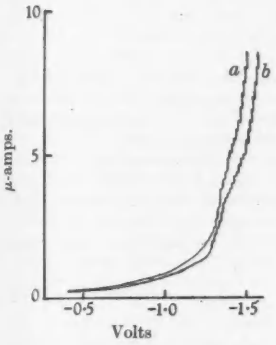

Fig. 2 *a*, *b*.

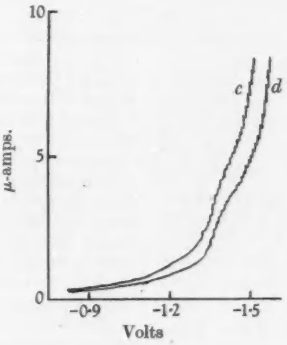

Fig. 2 *c*, *d*.

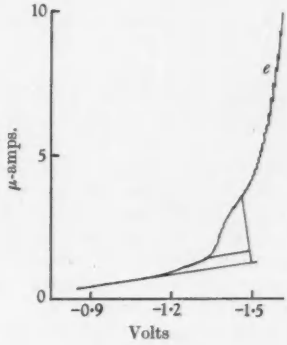

Fig. 2 *e*.

Fig. 2. Polarograms of neutral ketonic extracts. Sensitivity C. (Figs. *a*–*d* were obtained from the same urine.) *a*, crude extract + Girard reagent T (unspread). *b*, oxidized extract + Girard reagent T (unspread). *c*, crude extract + Girard reagent T (spread). *d*, oxidized extract + Girard reagent T (spread). *e*, oxidized extract + Girard reagent T (spread), showing method of assessing 'corrected value'.

SUMMARY

1. The polarographic method of estimating urinary neutral 17-ketosteroids ('urinary androgens') as described by Wolfe *et al.* (1940) has been confirmed and extended.

2. The technique of Werthessen & Baker (1945), whereby a decreased rate of increase of applied caused a deviation from the true base-line; a method for evaluating the true height of the wave has been adopted which, it would appear, measures only neutral 17-ketosteroids.

4. A series of estimations on 24 normal healthy young men has been carried out. The mean value found was 15·7 mg./24 hr., with a standard deviation of 2·47 mg.

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The Polarographic Estimation of Steroid Hormones

2. POLAROGRAPHY OF RELATED STEROID HYDRAZONES

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(*Received* 25 *February* 1946)

Parallel to the work described in Part 1 (Barnett, Henly & Morris, 1946), it was decided to investigate a series of substances capable of reacting with ketonic steroids to yield water-soluble compounds which might show more favourable polarographic waves than those obtained with the Girard T reagent. The disadvantages in the use of this reagent with urine extracts are twofold. First, with *iso*dehydroandrosterone the final discharge due to excess of the reagent occurs at about $-1{\cdot}5$ V., the half-wave potential of the steroid hydrazone being at $-1{\cdot}36$ V.; i.e. the range in which the flat part of the wave occurs is limited to 0·15 V. (see Fig. 1). Hence in urine extracts, where the wave tends to be much less sharply defined than with pure *iso*dehydroandrosterone, it often happens that the discharge due to the steroid hydrazones and that due to excess Girard T reagent form a practically continuous line, or show only a slight inflexion which cannot be accurately measured. It was hoped to find a hydrazine derivative whose steroid hydrazone might give a polarogram having a greater wave-spread, thus enabling the point of inflexion to be read more accurately. In the second place, polarograms of urine extracts generally show a deviation from the main base line between $-1{\cdot}0$ and $-1{\cdot}3$ V., before the discharge due to the steroid hydrazone takes place; this difficulty has already been discussed in detail (Barnett *et al.* 1946). It was hoped that use of a reagent which caused a considerable shift in the half-wave potential might eliminate this difficulty.

The substances chosen for testing were mainly acid hydrazides carrying a group which ensured water solubility of the steroid hydrazone. Besides these, aminoguanidine, nitroguanidine, thiosemicarbazide, hydrazinoacetic and hydrazinobenzoic acids were also tested.

EXPERIMENTAL

Melting-points are all uncorrected. Microanalyses by Drs Weiler and Strauss of Oxford.

Glycine hydrazide was prepared from freshly distilled glycine ester and hydrazine hydrate (60%) according to Curtius (1904).

Monomethylaminoacethydrazide. The method of Staudt (1925) was found the most satisfactory of several methods tried for the preparation of sarcosine. The hydrazide was prepared by refluxing sarcosine ester with 3·5 equiv. of hydrazine hydrate (60%) for 1 hr. The water was distilled off *in vacuo* and the residue dried overnight in a vacuum desiccator over sulphuric acid. The syrup was dissolved in absolute ethanol, treated with 2·2 mol. of ethanolic hydrogen chloride and the dihydrochloride recrystallized from water by addition of ethanol (m.p. 214–216°). Further recrystallization lowered the melting-point, apparently causing some decomposition. (Found: C, 20·84; H, 6·08; N, 23·8; Cl, 41·36. Calc. for $C_3H_{11}ON_3Cl_2$: C, 20·46; H, 6·29; N, 23·87; Cl, 40·28%.)

Dimethylaminoacethydrazide. Dimethylaminoacetic acid was prepared from glycine by the action of formic acid and formalin, according to Clarke, Gillespie & Weisshaus (1933) m.p. 187–192° from ethanol. The ethyl ester hydrochloride was prepared in the usual way and the free ester was obtained from it by means of silver oxide, b.p. 149–151°. The hydrazide was prepared as for sarcosine hydrazide. The syrupy hydrazide when rubbed with absolute ethanol gave a white solid which was recrystallized from ethanol, m.p. 209–210°. The dihydrochloride, prepared as above was recrystallized by dissolving it in the minimum amount of warm water and adding ethanol until the solution became cloudy, m.p. 214°. After four recrystallizations, m.p. 215°–216°. Hair-like needles, scarcely hygroscopic when pure. (Found: N, 21·80; Cl, 36·52. Calc. for $C_4H_{13}ON_3Cl_2$: N, 22·11; Cl, 37·31%.)

Maleic acid monohydrazide. Maleic acid monoethyl ester was prepared by refluxing the anhydride with absolute ethanol according to Sudborough & Roberts (1905). The syrupy residue after distilling off the ethanol *in vacuo* was treated with 1·5 mol. of hydrazine hydrate (60%), whereby much heat was evolved. The syrup was poured into a dish and left *in vacuo* over sulphuric acid for three days. The semicrystalline mass was rubbed with ethanol and filtered (m.p. *c.* 150°). It was recrystallized by dissolving in warm water and adding ethanol until it became cloudy. The monohydrazide crystallized as glistening leaflets which were filtered, washed with absolute ethanol and finally with dry ether (m.p. 154–155° (decomposition)). Melting-point after four further recrystallizations, 156–157°. (Found: C, 41·19; H, 6·70; N, 16·3%. Calc. for $C_4H_6O_3N_2 . C_2H_5OH$: C, 40·90; H, 6·86; N, 15·92%.) The monohydrazide is very soluble in cold water, sodium carbonate and cold glacial acetic acid, insoluble in ethanol and ether. It condensed readily with *iso*dehydroandrosterone (even at room temperature after a few minutes' standing appreciable condensation took place), to give a product which was completely water soluble.

Attempted preparation of itaconic acid monohydrazide. Itaconic anhydride was prepared according to *Organ.*

Synth. Coll. Vol. **2**, 368. The monoethyl ester, prepared from the anhydride by refluxing with ethanol, boiled at 134–136°/8 mm., and solidified in the receiver (Anschütz (1897*a*), gives b.p. 153°/12 mm.). The monoethyl ester has the structure 2-carboxy 3-carbethoxy Δ_1 propene, according to Anschütz (1897*b*). This monoester was treated with 1·5 mol. of hydrazine hydrate (60%), with cooling during the addition. After standing for 1 hr. at room temperature the syrup was left overnight in a vacuum desiccator over sulphuric acid. The semicrystalline mass was rubbed with ethanol until it became powdery, then was filtered, washed with ethanol and recrystallized from boiling water (m.p. 276–278° with charring). After two further recrystallizations the melting-point was raised to 277–278°. The material was soluble in hot water, insoluble in cold water, ethanol, ether, and glacial acetic acid. It dissolved readily in cold sodium carbonate solution. The high melting-point, and insolubility of the substance in water suggested that the product was not a hydrazide but had undergone ring closure. Analysis confirmed this. (Found: C, 47·67; H, 4·66; N, 22·20. Calc. for $C_5H_6O_2N_2$: C, 47·60; H, 4·80; N, 22·22. Calc. for $C_5H_8O_2N_2$: C, 41·67; H, 5·59; N, 20·00%.)

Hydrazinoacetic and p-*hydrazinobenzoic acids* were prepared as described by Anschel & Schoenheimer (1936). Hydrazinoacetic acid formed triangular, spear-headed plates, m.p. 151·5–152° from aqueous ethanol. *p*-Hydrazinobenzoic acid was obtained as yellow plates, m.p. 219° (decomposition).

p-*Aminobenzoic acid hydrazide* was prepared as described by Curtius (1917). After three recrystallizations from ethanol, it had m.p. 226–227° (Curtius (1917) gives m.p. 220°).

Dimethylaminobenzene sulphonhydrazide. Methyl *p*-dimethylaminobenzene sulphonate was prepared according to Houben & Schreiber (1920) as needles, m.p. 70° from aqueous ethanol. The sulphonhydrazide was prepared by warming the sulphonic ester with 3 mol. of hydrazine hydrate at 120°. For about 5 min. the reaction mixture remained in two layers, then reaction occurred suddenly with the formation of a homogeneous solution, which crystallized on cooling. Repeated crystallization from aqueous ethanol yielded two products, the more insoluble one having m.p. 118° with decomposition, and giving a blue coloration; this substance did not appear to react with *iso*dehydroandrosterone and was not further investigated; the second product, from the mother liquor, formed monoclinic prisms, m.p. 224–225° (decomposition); further recrystallization raised the m.p. to 226–227°; it formed hair-like needles readily soluble in cold water, insoluble in cold acetic acid, ethanol and ether. (Found: N, 18·3; S, 14·03. Calc. for $C_8H_{12}O_2N_2S.H_2O$: N, 18·02; S, 13·73%.)

Polarography. The general polarographic technique was identical with that previously described (Barnett *et al.* 1946). Half-wave potentials ($E_{\frac{1}{2}}$) were measured against the saturated calomel electrode at 25 ± 0·3°. The values of $E_{\frac{1}{2}}$ were obtained from curves at different concentrations and sensitivities. Change in concentration was found to have little if any effect on $E_{\frac{1}{2}}$.

RESULTS

Table 1 shows the substances which yielded products with *iso*dehydroandrosterone which gave a polarographic wave. All the polarograms were similar in shape to that obtained by Wolfe, Hershberg & Fieser (1940) using Girard T reagent. Fig. 1 shows the three most satisfactory polarograms obtained.

In addition, three further reagents were tested, with negative results, namely, hydrazinoacetic and hydrazinobenzoic acids and nitroguanidine. The first two substances themselves gave a wave either after a few minutes' heating in acetic acid at 100°, or after standing for some hours at room temperature, no change in the polarogram being evident after heating with the ketone. The third apparently gave no wave with *iso*dehydroandrosterone.

It will be observed that the half-wave potentials listed in the table fall within a very narrow range, except for those in which the two sulphur-containing analogues were used. It is to be noted that all these substances contain the common linkage

$$-\overset{\|}{C}-NH-NH_2$$

with the possibility of enolization on the carbon atom adjacent to the imide nitrogen atom, thus enabling a conjugated system to be formed when reaction with the ketone takes place. The unusually high reactivity of maleic acid monohydrazide is probably due to activation of the hydrazide linkage by the terminal carbonyl group, transmitted through the conjugated system. It would therefore appear likely that any substance capable of reacting with a ketone to form the grouping

$$R-\underset{|}{X}=N-N=R',$$

where R may be —OH (as in the acid hydrazides), —NH_2 (as in aminoguanidine), —SH (as in thiosemicarbazide) and X=C or S, will show a polarographic wave similar in shape to that of derivatives obtained with the Girard T reagent. Hydrazinoacetic and hydrazinobenzoic acids, which do not conform to the above structure, apparently gave no wave after reaction with the ketone.

It is apparent that substitution in the part of the reagent molecule not concerned in this linkage has very little effect on the half-wave potential, even when it involves a marked change in the polarity of the molecule as in the series of methyl-substituted glycine hydrazides. The only noticeably different figures were obtained with thiosemicarbazide and the sulphonhydrazide, both of which were too insoluble in acetic acid to be of practical value.

Moreover, in no case was the desired 'spread' in the wave pattern obtained. Such cases as showed a lower half-wave potential showed also a lower discharge potential for the reagent itself, so that one of the advantages sought for in changing the half-wave potential of the ketone-complex was lost.

Table 1. *Results of polarography of steroid hydrazones*

Reagent	Conditions for reaction of substance with *iso*dehydroandrosterone	$E_{\frac{1}{2}}$ measured against calomel electrode (volt)	Wave span for 100 μg. *iso*dehydroandrosterone at sensitivity C (Barnett *et al.* 1946) (mm.)
Glycine hydrazide	Heating for 2 min. at 100° in acetic acid	−1·32	20
Monomethylamino-acethydrazide	As for glycine hydrazide	−1·36	10·5
Dimethylamino-acethydrazide	30 min. at 20° in acetic acid. Substance gave a wave by itself when heated for 5 min. at 100°	−1·39	9·5
Trimethyl acethydrazide ammonium chloride (Girard T)	As for glycine hydrazide	−1·35	20
Maleic acid monohydrazide	30 min. at 80° in acetic acid. Gave a wave by itself when heated at 100° for 5 min.	−1·32	20
Aminoguanidine	5 min. at 100° or 1 hr. at 20°, when freshly made solution in acetic acid is used, but after solution has stood for 24 hr. it gives no wave	−1·41	14
Thiosemicarbazide	5 min. at 100°. But solubility in acetic acid is very low. Small wave span therefore probably due to incomplete reaction	−0·9*	3·1
p-Aminobenzoic acid hydrazide	2 min. at 100° in acetic acid	−1·25	7·5
p-Dimethylaminobenzene sulphonhydrazide	5 min. at 100°. Solubility in acetic acid low, as for thiosemicarbazide	−1·09*	6·2

* These values were measured against the mercury pool.

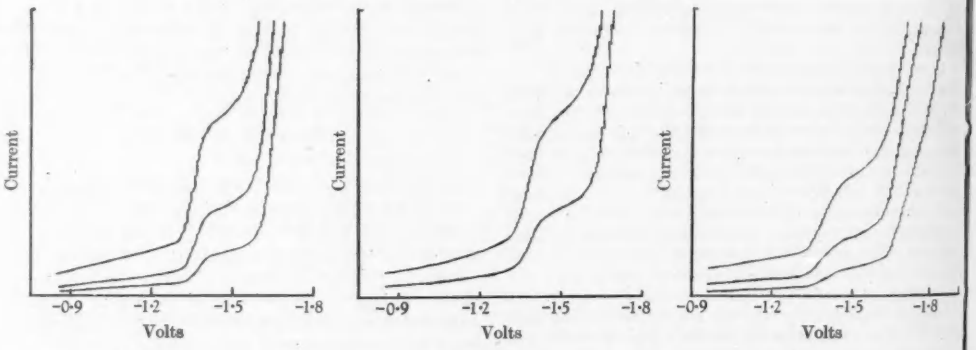

Fig. 1 *a*. Fig. 1 *b*. Fig. 1 *c*.

Fig. 1. Polarograms of *iso*dehydroandrosterone hydrazones. 100 μg. *iso*dehydroandrosterone, after treatment with: Girard reagent T (1 *a*), maleic acid monohydrazide (1 *b*), and aminoguanidine (1 *c*), at sensitivities B, C and D (Barnett *et al.* 1946).

The only two substances tested which appeared as possible practical alternatives to Girard T reagent were maleic acid monohydrazide and aminoguanidine. However, these reagents appeared to offer no advantages over the Girard T reagent for polarographic usage.

SUMMARY

1. A number of new reagents, mainly acid hydrazides, has been prepared which react with *iso*dehydroandrosterone to yield water-soluble compounds.

2. In all cases the polarograms of the steroid hydrazones were similar in shape to that obtained from the condensation product of *iso*dehydroandrosterone and Girard T reagent. The half-wave potentials were practically unaltered except in two cases.

3. A chemical structure responsible for the characteristic wave form is suggested.

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The Effect of Phytic Acid on the Absorption of Calcium and Phosphorus

2. IN INFANTS

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(*Received* 31 *January* 1946)

It appears from previous experiments (Hoff-Jørgensen, 1946) that in very young puppies the decrease in calcium absorption caused by the presence of phytic acid in the diet is only moderate, while it is considerable in somewhat older puppies. In order to see whether children react in a similar manner experiments were done in which infants were given a diet containing varying amounts of phytic acid.

EXPERIMENTAL

Subjects

The investigation was carried out between September and November 1944 with four male infants who at the beginning of the experiment were 1, 4, 6 and 11 months old. The infants were admitted to hospital for different reasons, and at the time of the experiment their organic diseases had been cured, and they were only detained at hospital for the sake of the experiment. In particular they presented no signs of dyspepsia or of rickets; their blood P, Ca and phosphatase were normal. Further particulars are given in Table 1.

Diet

The composition of the diets is given in Table 1. Cow's milk was used throughout. During the phytate periods a solution of sodium phytate (*c.* 25%, pH 6·8) was added to all the milk. Thereby a sparingly soluble calcium phytate was formed, which by analogy with that formed in pure aqueous solutions has the composition $C_6H_8O_{24}P_6Ca_5$ (Hoff-Jøgensen, 1944). The suspension of calcium phytate precipitated in milk is very stable.

Table 1. *Particulars of infants and of diet* and treatment*

Infant	Date of Birth	Date of Admission	Weight	Complaint on admission	Treatment	Diet during experiment
B.	19. ix. 44	22. ix. 44	4·0 kg. at 1·5 months	Admitted for vaccination with B.C.G.	No medical treatment. No additional vitamins	Milk and water 1:1 with 2% sucrose
N.N.	25. v. 44	16. viii. 44	4·2 kg. at 4 months	*Dyspepsia antea*	Carrot soup. No additional vitamins	Semolina gruel; butter-milk gruel; fruit juice
E.P.	6. v. 44	16. ix. 44	6·4 kg. at 5 months	*Dyspepsia antea*	Carrot soup. 600 i.u. vitamin D_3 and 100 mg. ascorbic acid daily	As above
H.C.S.	20. x. 43	23. viii. 44	9·1 kg. at 12 months	Imbecility	No medical treatment. No additional vitamins	As above; rusks

* During the phytate periods a solution of sodium phytate (*c.* 25%, pH 6·8) was added to the milk; see also Table 2.

Table 2. *The effect of phytate on the retention of* Ca *and* P *in four infants*

Duration of exp. (days)	Total intake of P (mg.)	Total intake of Phytate-P (mg.)	Total intake of Ca (mg.)	Total excretion of P in faeces (mg.)	Total excretion of P in urine (mg.)	Total excretion of Ca in faeces (mg.)	Total excretion of Ca in urine (mg.)	Total excretion of Phytate-P in faeces (mg.)	Daily retention of Ca mg.	Daily retention of Ca %	Daily retention of P mg.	Daily retention of P %
Subject B. Age 1–1·5 months												
3	1078	0	1206	334	212	563	22	0	207	51·5	177	49·3
3	2105	947	1286	814	321	1285	19	536	−6	—	323	46·1
3	2392	1194	1266	945	445	1395	17	758	−49	—	334	42·6
Subject N.N. Age 4–5 months												
4	2772	0	3062	592	1184	2164	22	0	219	28·6	249	35·9
3	2448	0	2533	894	872	1826	28	0	226	26·8	227	27·9
3	3067	942	2120	1112	1196	1892	12	612	72	10·2	253	24·8
3	5502	2680	2822	2282	1645	3541	0	1762	−240	—	525	28·6
Subject E.P. Age 6–7 months												
3	2380	0	2452	493	1246	1842	21	0	196	24·0	214	27·0
3	5099	1762	3632	1987	1794	3185	17	970	143	11·9	439	25·8
3	6166	3062	3314	3662	1256	3821	10	2142	−172	—	416	20·2
Subject H.C.S. Age 11–12 months												
3	1826	0	1628	280	882	772	63	0	264	48·7	221	36·4
3	2084	0	1989	438	1065	1425	33	0	177	26·7	194	27·9
2	2680	1208	1146	1115	1021	1632	5	642	−246	—	272	20·3

Collection and analysis of excreta

Faeces were collected in napkins in which were placed pieces of rubber cambric from which faeces could be removed almost quantitatively. Carmine was used as a faecal marker to separate periods. The urine was collected in glass urinals. The analyses of diet, faeces and urine were carried out as previously described (Hoff-Jørgensen, 1946).

Results

The results will be found in Table 2.

DISCUSSION

The results of these experiments on infants agree with those of McCance & Widdowson (1942) on adults in showing that the addition of sodium phytate to the diet decreases the calcium absorption very considerably. The effect of phytate on calcium absorption seems to be the more pronounced the older the infants; this accords with observations on puppies (Hoff-Jørgensen, 1946), but the effect is less conspicuous in the infants. The addition of phytate to the diet causes an increased absorption of phosphate. In some of the experiments the amount of phosphorus absorbed was greater than the amount of non-phytate phosphorus in the diet. Some of the phosphate, which was split from the phytate in the intestine (about 35% of the intake) must therefore have been absorbed. Two infants (N.N. and E.P.) were given the same diet during the experiment, except that E.P. received daily a supplement of 600 i.u. of vitamin D_2 and of 100 mg. of ascorbic acid. The inhibitory effect of phytate on the calcium absorption was slightly less in E.P. than in N.N., but the difference was not significant enough to prove an effect of the vitamins.

SUMMARY

The addition of sodium phytate in amounts sufficient to combine with about 50, 75 and 100% of the total calcium of a diet containing cow's milk caused in infants a very considerable decrease in the calcium absorption and an increase in the phosphate absorption.

The above work was part of the programme of the Cereal Committee of the Academy of Technical Sciences, Copenhagen.

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The Quantitative Separation of Trivalent from Pentavalent Arsenic Derivatives on the Micro Scale

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(*Received* 29 *March* 1946)

Sodium ethyl xanthate dissolved in carbon tetrachloride has been shown to be effective for the quantitative separation of arsenious acid from arsenic acid in pure solution, blood or urine (Crawford & Storey, 1944), and has been used to determine the valency of the arsenic compounds excreted in the urine after injection of sodium arsenite or sodium arsenate (Storey, 1944) and in blood after the inhalation of arsine (Graham, Crawford & Marrian, 1946).

It was planned to use this reagent in studies of the metabolism of phenylarsenious acid and phenylarsonic acid. It was found, however, in experiments carried out in pure solution, that the xanthate reagent was unsuitable for the separation of phenylarsenious acid from phenylarsonic acid.

Quantitative removal of phenylarsenious acid, but not of phenylarsonic acid, from aqueous solutions was obtained using a solution of ethane-1:2-dithiol in carbon tetrachloride (dithiol reagent) in place of the xanthate reagent. The use of a 1:2-dithiol for this purpose was suggested by the work of Peters, Stocken & Thompson (1945), who showed BAL (2:3-dimercaptopropanol) and related compounds to be effective antidotes in arsenical poisoning, in virtue of forming stable compounds with trivalent arsenic derivatives. The choice of dithiol in the initial experiments was determined by the fact that ethane-1:2-dithiol was available, having been used by one of the authors as an antidote in arsine poisoning (Levvy, quoted by Peters *et al.* 1945). The dithiol reagent was also found to be efficient for the quantitative separation of arsenious acid from arsenic acid and more convenient to use than the xanthate reagent. Later the use of BAL itself for separating tri- and pentavalent arsenicals was examined, but without success.

EXPERIMENTAL

General methods

Standard solutions of phenylarsenious acid, phenylarsonic acid and arsenious acid were prepared by dissolving weighed quantities of the solids in aqueous NaOH and, after adjusting the pH to neutrality (glass electrode) by the addition of HCl, diluting to the required volume. Arsenic acid solutions were prepared by dissolving $Na_2HAsO_4.7H_2O$ (analyzed specimen) in water, followed by pH adjustment. Solutions of all four compounds were checked from time to time for partial oxidation or reduction by direct iodometric titration.

Fractionations were carried out in 150 ml. separating funnels, on approximately 40 ml. batches of arsenic-containing solution, which were always first acidified by addition of 20 ml. 18 N-H_2SO_4. Arsenic was estimated throughout by the method of Levvy (1943) using the 'small-scale' procedure. Total arsenic in the original solutions employed was determined simultaneously with the analysis of the samples from the fractionation, and the figures thus obtained used in calculating the recovery in each fraction. This eliminated, to some extent, constant errors arising from the arsenic determinations. Control fractionations without added arsenic were carried out concurrently with all experiments, and corrections for arsenic in reagents applied to all results.

RESULTS

Use of sodium ethyl xanthate for the separation of phenylarsenious acid from phenylarsonic acid in simple solution

Solutions of phenylarsenious acid and of phenylarsonic acid were extracted with the xanthate reagent according to the method of Crawford & Storey (1944). It will be seen (Table 1) that phenylarsenious acid was not quantitatively extracted

Table 1. *Extraction of phenylarsenious acid and phenylarsonic acid from aqueous solution with sodium ethyl xanthate in carbon tetrachloride*

Age of stock reagent (days)	Total As present (μg.)	As removed from aqueous layer (%)
Phenylarsenious acid		
Fresh	80	90
Fresh	80	90
Fresh	77	85
7	79	92
Phenylarsonic acid		
Fresh	92	8
Fresh	88	2
Fresh	92	5
Fresh	89	7
Fresh	90	8
7	92	19
10	90	33
10	90	34

from the aqueous solution. Increasing the number of the extractions did not affect the final result. Phenylarsonic acid was always partially reduced, and then extracted, to an extent dependent upon the age of the stock xanthate solution.

Use of the dithiol reagent for the separation of phenylarsenious acid and arsenious acid from phenylarsonic acid and arsenic acid in simple solution

Ethane-1:2-dithiol was prepared according to the method of Fasbender (1887). The pure substance appears to be stable for several months at least. For the 'dithiol reagent', which was made up each day, 4 ml. ethane-1:2-dithiol were diluted to 1 l. with carbon tetrachloride. The solution of the arsenical was acidified and extracted by vigorous shaking for 2 min. with two 20 ml. portions of the dithiol reagent followed by three 20 ml. portions pure carbon tetrachloride. Between each fresh treatment with the dithiol reagent and carbon tetrachloride, 5 ml. carbon tetrachloride was added to the funnel and run off without any shaking in order to wash through the stopcock and stem of the funnel. The back-washing of the combined extracts with $2N\text{-}H_2SO_4$, recommended for the xanthate reagent, was dispensed with as it was found to be without effect on the results when the dithiol reagent was used.

Table 2. *Extraction of phenylarsenious acid, phenylarsonic acid, arsenious acid and arsenic acid from aqueous solution with ethane-1:2-dithiol in carbon tetrachloride*

(Figures shown are means of triplicate experiments)

Total As present (μg.)	As removed from aqueous layer (%)
Phenylarsenious acid	
66	95
99	97
9·8	89
Phenylarsonic acid	
79	Nil
75	1
89	4
8·8	8
Arsenious acid	
67	100
68	100
94	101
9·4	102
Arsenic acid	
82	Nil
73	4
70	Nil
70	Nil
75	2
82	Nil
68	Nil
91	3

Arsenic was determined in the aqueous phase, and in the combined extracts after removal of the carbon tetrachloride by distillation at normal pressure in the presence of 25–30 ml. 1% Na_2CO_3 solution. The use of alkali is necessary to retain the dithiol derivative of phenylarsenious acid which was found to be steam-volatile.

The results in Table 2, which are the means of experiments carried out in triplicate, show that the

Table 3. *Separation of arsenious and arsenic acids*

(1) Separation of phenylarsenious acid from phenylarsonic acid, in simple solution, with ethane-1:2-dithiol in carbon tetrachloride (20 experiments)

	Phenylarsenious acid	Phenylarsonic acid
Range of As added (μg.)	9·8–90·6	8·8–89·0
Range of As found (μg.)	10·1–87·1	9·1–87·3
Mean error (μg. As)	−2·1	−0·9
S.D. of the error (μg. As)	6·3	2·2

(2) Separation of arsenious acid from arsenic acid, in simple solution, with ethane-1:2-dithiol in carbon tetrachloride (24 experiments)

	Arsenious acid	Arsenic acid
Range of As added (μg.)	9·3–105·0	9·3–92·4
Range of As found (μg.)	10·9–94·0	6·4–100·2
Mean error (μg. As)	−0·4	−0·9
S.D. of the error (μg. As)	6·3	3·9

(3) Separation of phenylarsenious acid from phenylarsonic acid, in urine dialysate, with ethane-1:2-dithiol in carbon tetrachloride (17 experiments)

	Phenylarsenious acid	Phenylarsonic acid
Range of As added (μg.)	9·8–90·6	8·8–95·0
Range of As found (μg.)	11·0–99·6	5·0–84·3
Mean error (μg. As)	+2·0	−2·6
S.D. of the error (μg. As)	4·0	6·3

(4) Separation of arsenious acid from arsenic acid, in urine dialysate, with ethane-1:2-dithiol in carbon tetrachloride (11 experiments)

	Arsenious acid	Arsenic acid
Range of As added (μg.)	9·3–97·0	9·6–88·8
Range of As found (μg.)	13·1–94·2	4·0–91·6
Mean error (μg. As)	+1·5	−0·1
S.D. of the error (μg. As)	5·6	2·9

dithiol reagent extracted the trivalent arsenic compounds from acidified aqueous solution quantitatively while little or none of either pentavalent compound was removed.

The mean error and the standard deviation of the error for a single observation from the mean error in experiments on the separation of phenylarsenious acid from phenylarsonic acid and of arsenious acid from arsenic acid in simple solution are given in Table 3. Since the error appeared to be approximately constant over the whole range studied, it is given as μg. arsenic and not as a percentage.

Use of the dithiol reagent for the separation of phenylarsenious acid from phenylarsonic acid and of arsenious from arsenic acid when present together in urine dialysates

A 24 hr. specimen of rabbit urine, collected over 2 ml. chloroform, was dialyzed in a Visking sausage casing sac against approximately 300 ml. distilled water containing a trace of chloroform, for 24 hr. in the refrigerator. After addition of known amounts of the arsenicals and acidifying, samples of the dialysate were extracted with the dithiol reagent as described above.

The mean error and the standard deviation of the error for a single observation from the mean error in experiments on the separation of phenylarsenious acid from phenylarsonic acid and of arsenious acid from arsenic acid in urine dialysate are given in Table 3.

BAL (2:3-dimercaptopropanol) as a reagent for the separation of trivalent from pentavalent arsenicals

The inability of BAL, dissolved in carbon tetrachloride or chloroform, to effect a separation of the trivalent from the pentavalent arsenicals is illustrated in Table 4. Solutions of each of the four arsenicals used in the experiments described above, after acidification, were extracted with a solution of BAL as indicated in the table of results, the separating funnel stopcock and stem being washed down with 5 ml. of the appropriate solvent between each fresh treatment with the BAL solution.

With BAL in carbon tetrachloride, phenylarsenious acid was completely removed from aqueous solution while arsenious acid was only partially extracted. The latter observation is probably to be explained by an unfavourable partition of the arsenious acid derivative of BAL between carbon tetrachloride and water. The pentavalent arsenicals behaved towards the reagent in exactly the same way as the corresponding trivalent compounds, suggesting that BAL brought about preliminary reduction of the pentavalent arsenicals.

Table 4. *Extraction of phenylarsenious acid, phenylarsonic acid, arsenious acid and arsenic acid from simple solution by BAL (2:3-dimercaptopropanol)*

(Figures shown are means of triplicate determinations)

Arsenical	Total As present (μg.)	As removed from aqueous layer (%)
Extraction with one 40 ml. and two 20 ml. portions of BAL in carbon tetrachloride (0·4% v/v)*		
Phenylarsenious acid	81	98
Phenylarsonic acid	83	100
Arsenious acid	76	48
Arsenic acid	93	51
Extraction with two 60 ml. and one 40 ml. portions of BAL in carbon tetrachloride (0·1% v/v)		
Phenylarsenious acid	80	100
Phenylarsonic acid	85	100
Arsenious acid	80	66
Arsenic acid	80	50
Extraction with two 60 ml. and one 40 ml. portions of BAL in chloroform (0·2% v/v)*		
Phenylarsenious acid	82	100
Phenylarsonic acid	85	100
Arsenious acid	65	100
Arsenic acid	110	100

* At this concentration, all the BAL did not dissolve.

The use of chloroform instead of carbon tetrachloride as a solvent for the BAL, gave complete extraction of the arsenious acid derivative of BAL from aqueous solution, but the pentavalent compounds again reacted with the dithiol.

DISCUSSION

Sodium ethyl xanthate in carbon tetrachloride, while an effective agent for the separation of arsenious acid from arsenic acid, does not give a clear distinction between the corresponding unsubstituted phenyl derivatives, phenylarsenious acid and phenylarsonic acid, the former being only partially removed from aqueous solution, while the latter reacts with the reagent to an extent dependent on its age. Ethane-1:2-dithiol in carbon tetrachloride has been found very satisfactory for the separation of phenylarsenious acid from phenylarsonic acid and of arsenious acid from arsenic acid in aqueous solutions and urine dialysates. While the error in the procedure may be considerable with very small amounts of arsenic, this does not appear to be due to incomplete separation of the arsenicals as only rarely was an error in the one fraction offset by a corresponding error of opposite sign in the other fraction. It should be noted that the standard deviation of a single observation in the method used for the arsenic determinations is of the order of 12% (Levvy, 1943). BAL (2:3-dimercaptopropanol) was

found to be unsuitable for the separation of trivalent from pentavalent arsenicals as it reduced the latter.

The use of the dithiol reagent has been incorporated successfully into a method whereby the amounts of each component of a mixture of phenylarsenious acid, arsenious acid, phenylarsonic acid and arsenic acid may be determined. It is hoped to publish in the near future details of this method, along with its application to studies of the metabolism of phenylarsenious acid and of phenylarsonic acid.

SUMMARY

1. Sodium ethylxanthate in carbon tetrachloride, while an effective agent for the separation of arsenious from arsenic acid, is unsuitable for the separation of the corresponding unsubstituted phenyl derivatives, phenylarsenious acid and phenylarsonic acid.

2. Quantitative separation of these organic arsenicals in acid solution as well as the separation of arsenious and arsenic acids, is obtained by the use of a 0·4% (v/v) solution of ethane-1:2-dithiol in carbon tetrachloride.

3. BAL (2:3-dimercaptopropanol), dissolved in carbon tetrachloride or chloroform, does not differentiate between the trivalent and the pentavalent arsenicals.

The authors wish to express their gratitude to the Chief Scientific Officer, Ministry of Supply, for permission to publish this work.

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The Interdependence of the Vitamins. Vitamin B_1 and Riboflavin

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(*Received* 3 *April* 1946)

Evidence has gradually accumulated that the response of an animal to a dose of one vitamin may be influenced by the amount of another vitamin present either in the body reserves or in the diet of the animal. A partial deficiency of the vitamin in reserve or diet will lower the response of the animal not only to high doses, but also to low doses of the vitamin under test (Sherman & Batchelder, 1931; Coward, Key & Morgan, 1932; Coward, Burn, Ling & Morgan, 1933; Coward, Key, Dyer & Morgan, 1930; Moore, 1940; Hickman, Kaley & Harris, 1944*a*, *b*, *c*).

The evidence was obtained from two types of experiment: (*a*) after suitable depletion, all animals were given the same small dose of a vitamin and distributed into different groups which received graded doses of the other vitamin. Up to a certain point, probably the maximum, the response to a given dose of the one vitamin depended on the amount of the other vitamin given also (Hickman *et al.* 1944*a*, *b*, *c*); (*b*) comparison was made between the spread of the composite growth curves of groups of rats which had received a limited amount of one vitamin and graded amounts of another and the spread of other growth curves of rats which had received a much larger dose of the one vitamin and similarly graded doses of the other, i.e. the response/log dose curves were compared for slope and found to be steeper when larger amounts of the first vitamin were given to all rats.

The second type of experiment is certainly more convincing than the first but both would surely be considered by Fisher (1942) as extravagant in time, labour and material when considered in relation to the amount of information obtained. A more economical and much more informative experiment could be carried out on the plan proposed by Fisher for investigating the influence of different amounts of different fertilizers, singly and combined with each other, on crops of cereals, potatoes, etc.

A very simple experiment was designed on this plan and carried out in this laboratory to investigate not only the influence of one vitamin on a second but at the same time the influence of the second vitamin on the first. By the same design, the possible interaction of the two vitamins was

investigated. A similar design could be applied to determine the interrelationship and interaction of three or more vitamins.

EXPERIMENTAL

Methods

Eighty rats each weighing 40–50 g. and 4–5 weeks of age were selected from litters containing four males or four females each and distributed into four groups of ten males and ten females in each, each litter being equally represented in all groups. Each rat was housed in a separate cage provided with a grid of ½ in. mesh to prevent access to faeces. The diet of the rats consisted of:

Casein (Glaxo, ashless extracted)	15%
Rice starch, partially dextrinized	81%
Salt mixture (Steenbock's 40)	4%

Five drops of a good sample of cod-liver oil per rat twice a week.

0·5 ml. liver extract, norite treated, equivalent to 5 g. whole liver, per rat per day, prepared in the Glaxo Laboratories.

The whole liver extract was filtered, extracted with ether, and the residual ether removed *in vacuo*. The extract (1 ml. equiv. to 5 g. liver) was adjusted to pH 5 and treated twice with charcoal (15 g./l. the first time, 10 g./l. the second). The filtrate and washings were concentrated *in vacuo* till 1 ml. was equivalent to 10 g. of original liver.

The rats of the respective groups were given daily from the beginning of the experiment:

Group I: 1 i.u. vitamin B_1 + 2·5 μg. riboflavin
Group II: 2 i.u. vitamin B_1 + 2·5 μg. riboflavin
Group III: 1 i.u. vitamin B_1 + 5·0 μg. riboflavin
Group IV: 2 i.u. vitamin B_1 + 5·0 μg. riboflavin

RESULTS

The rats were weighed once a week, and the test was carried on for 5 weeks. At the end of that time, the increases in weight of the rats in each group were averaged. It was found that the averages for the male and female rats of each group differed only slightly and not always in the same direction so the sexes were not treated separately. The average increases in weight may be tabulated thus:

Doses given (μg. riboflavin)	1 i.u. vitamin B_1 (g.)	2 i.u. vitamin B_1 (g.)
2·5	24·85	29·25
5·0	29·70	35·40

Simply stated, the result showed that:

(1) Doubling the dose of vitamin B_1 had some effect but it had a greater effect when the dose of riboflavin also was doubled.

(2) Doubling the dose of riboflavin had some effect but it had a greater effect when the dose of vitamin B_1 also was doubled.

Thus the interdependence of the two vitamins was demonstrated simultaneously in one experiment, at something like half of the expenditure of time, labour and material that would have been necessary if each had been investigated separately.

The interaction of the vitamins

An experiment designed as this one was, not only demonstrates the dependence of one vitamin on another for exerting its full influence but affords a means of finding out whether the vitamins interact with each other. If there had been interaction in the experiment then the slope of the response/log dose curve from 1 and 2 i.u. vitamin B_1 should have been steeper when 5 μg. of riboflavin were given to all the rats than it was when only 2·5 mg. riboflavin were given. Also the slope of the response/log dose curve from 2·5 and 5·0 μg. riboflavin should have been steeper when 2 i.u. of vitamin B_1 were given than when only 1 i.u. was given. As the log dose interval was the same (0·3010) in all the four curves mentioned, the significance of the difference in slope can be (for the sake of simplifying the arithmetic) worked out on the differences in average increases in weight.

An analysis of variance gave the following results:

	Sum of squares of deviations	Degrees of freedom	Mean square	Variance ratio
Error due to difference between doses	1123·5	3	374·50	9·057 (0·1% pt. = 6·17)
Error due to difference between litters	1040·3	19	54·75	1·324 (5% pt. approx. = 1·79)
Error due to randomization	2339·0	57	41·35	
	4502·8	79		

Thus $s^2 = 41{\cdot}35$; $s = 6{\cdot}43$.

Comparison of slopes obtained from Groups I and II and from Groups III and IV (based on increases in weight without dividing by log dose interval):

$$\frac{(35{\cdot}40 - 29{\cdot}70) - (29{\cdot}25 - 24{\cdot}85)}{\sqrt{\left(\frac{41{\cdot}35}{40} + \frac{41{\cdot}35}{40}\right)}} = \frac{1{\cdot}30}{1{\cdot}4381} = 0{\cdot}904,$$

which is certainly not significant. Similarly:

Comparison of slopes obtained from Groups I and III and from Groups II and IV:

$$\frac{(35{\cdot}40 - 29{\cdot}25) - (29{\cdot}70 - 24{\cdot}85)}{\sqrt{\left(\frac{41{\cdot}35}{40} + \frac{41{\cdot}35}{40}\right)}} = \frac{1{\cdot}30}{1{\cdot}4381} = 0{\cdot}904,$$

which again is not significant.

Thus the curves of response to doses of vitamin B_1 are not of significantly different slope when the doses of riboflavin are different and also the curves of response to doses of riboflavin are not of significantly different slope when the doses of vitamin B_1 are different. That is, with the rather small doses of vitamins employed in this experiment, there was no evidence of interaction of vitamin B_1 and riboflavin as judged by the increase in weight of the rat.

SUMMARY

1. The design is described of an experiment for testing (*a*) the interdependence of two vitamins (B_1 and riboflavin) in their influence on the increase in weight of the rat and (*b*) their possible interaction.

2. It was found that doubling the dose of either vitamin while keeping the dose of the other constant resulted in a significantly greater increase in weight of the rats. Doubling the dose of both vitamins resulted in a still greater increase in weight. Thus the response to one vitamin may be limited by the amount of another one given at the same time.

3. By an analysis of variance and by comparing the slopes of the response/log dose curves, it was found that the slopes of the vitamin B_1 curves were not significantly different when different amounts of riboflavin were given. The same was true of riboflavin in the presence of different amounts of vitamin B_1. Thus it was shown that with the small doses of vitamins used in this experiment there was no interaction of vitamin B_1 and riboflavin in their influence on the increase in weight of rats.

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The Metabolism and Functioning of Vitamin-like Compounds

3. PRODUCTS OF THE DECOMPOSITION OF GLUTAMINE DURING STREPTOCOCCAL GLYCOLYSIS

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(*Received* 22 *March* 1946)

Interest in the present reaction lies in the connexion which it may afford between a biological response of haemolytic streptococci to glutamine, and a metabolic response. The biological effect is a stimulation of growth (McIlwain, Fildes, Gladstone & Knight, 1939) and the metabolic one an increase in rate of glycolysis. While glycolyzing, the streptococci form a volatile base from added glutamine; but if glucose is not present—or if, while present, its breakdown is prevented—the reaction with glutamine does not occur (McIlwain, 1946). The products of the reaction have now been found to be glutamic acid and ammonia. As the reaction differs from that reported for some glutaminases in its dependence on glycolysis, it has been examined quantitatively, by several methods, for evidence of any chemical connexion with glucose or products of glycolysis. Also, attempts have been made to modify or couple the reaction by providing additional substances which might take part in further reactions with NH_3.

EXPERIMENTAL

Organisms. Certain of the strains of haemolytic streptococci used in Part 1 (McIlwain, 1946) were employed in the present work; they were grown as there described, but were maintained by drying newly passaged cultures *in vacuo*.

After recovery from mice, organisms were grown in blood-broth for 5 hr. and a drop of the culture pipetted to each of a number of ampoules. These were dried at room

temperature over P_2O_5 at 0·5 mm. Hg and sealed at that pressure. A fresh ampoule was opened each 10–14 days, its organisms grown for 5 hr. in blood-broth and the culture afterwards kept at 0°. Experimental material was grown from an inoculum of one loop of such a culture.

Metabolism. Organisms were collected by centrifuging and material from a culture of 100 ml. washed twice with saline. The material was then suspended in saline and measured samples normally placed in the sidearms of Warburg vessels of 15–20 ml. or of 100 ml. if several determinations were being made. The main compartments of the vessels contained glucose, 0·067 M; $NaHCO_3$, 0·05 M; glutamine, 0·0083 M, and the inorganic salts of Krebs & Eggleston (1940), with, however, only 0·001-M phosphate. Centre pots contained yellow phosphorus and the gas space, 5% CO_2 in N_2. Organisms were added to the metabolites at 37°, and the glycolysis followed manometrically. Reaction products were determined without separating the bacteria, which contributed relatively little of the substances concerned. Suspensions of the 'Richards' *Streptococcus* as used for the metabolic experiments were found to contain (μmol./mg. dry wt.): NH_3, 0·05; glutamine, 0·05, 0·09; 'amino-N' after 5 min. reaction with nitrite, 0·60, 0·71; after 20 min. reaction, 0·81.

Large-scale experiments from which glutamic acid was isolated were carried out with sterile precautions in a 250 ml. flask at 37° in a slow stream of N_2 with 5% CO_2, but without other attempt at anaerobiosis. Bicarbonate (M/2) was added during fermentation, to maintain the pH at 7–7·5.

l-(+)-Glutamine was purified as previously described (McIlwain, 1946). As arginine has been recorded as an unnoticed impurity in specimens of natural glutamine and as the present organisms readily liberate NH_3 from arginine, any of this substance present in a specimen of glutamine was separated by Archibald's (1945*a*) method; but no change was observed in the streptococcal reaction to the material. Glutamine was determined as amide-NH_3, labile under the conditions of Vickery, Pucher, Clark, Chibnall & Westall (1935) (cf. McIlwain, 1946).

Ammonia was determined after distillation *in vacuo* in a Parnas apparatus, usually acidimetrically. When estimated by Nessler's reagent, hypochlorite (Barrett, 1935) was added to the reagent described by Peters & Van Slyke (1932), and colour intensities were determined with a photoelectric absorptiometer.

'Amino-N.' Peters & Van Slyke's (1932) method and apparatus were used. Before carrying out determinations in solutions containing NH_3, this was removed by adding a borate buffer of pH 9 and evaporating twice *in vacuo* at 40°. This was shown not to cause decomposition of glutamine and to have little effect on the 'amino-N' of the bacteria. Glutamine yielded, in the concentrations in which it was being determined in mixtures from bacterial metabolism, 1·9 mol. of 'amino-N' per mol.

Substances yielding succinic acid after treatment with chloramine-T, and acid hydrolysis. Cohen's (1939) method for determination of glutamic acid was applied. In the present reaction mixtures, the yield of succinic acid from glutamic acid was 95–100%, and from glutamine 55–65% (cf. Cohen, 1939; Örström, Örström, Krebs & Eggleston, 1939). Chloramine-T did not liberate CO_2 from pyrrolidone-α-carboxylic acid, and no succinic acid was yielded by the subsequent treatment.

Separation of glutamic acid and glutamine. Repetition of Foreman's (1914) precipitation gave the necessary separation; the following experiment was designed to simulate circumstances anticipated after a streptococcal reaction with glutamine.

To 300 μmol. ammonium *l-(+)*-glutamate and 100 μmol. *l-(+)*-glutamine in 10 ml. water cooled by ice, were added 0·5 ml. 10% $CaCl_2$ and excess of a 20% suspension of $Ca(OH)_2$. The mixture was rubbed occasionally during 20 min., filtered, washed, and 60 ml. ethanol added to the filtrate and washings. A precipitate formed and was separated after 2 hr. at 0° and washed with 80% ethanol, dissolved in water, made to 10 ml., an aliquot kept for determination of glutamine and NH_3, and the bulk re-precipitated as above.

Both precipitates contained negligible NH_3; the first precipitate had 11·7 μ mol. of glutamine and the second, 1·9. Calcium was removed from the remainder of the second precipitate with oxalic acid, the solution evaporated to crystallization and ethanol added to 75% v/v. The yield of glutamic acid corresponded to 207 μmol. in the whole (70% of that added).

Recovery of glutamic acid from mixtures after streptococcal glycolysis. When glutamine was known to be absent, Neuberg & Kerb's (1912) precipitation as the Hg salt of the carbamido derivative was employed; the following experiment showed its applicability.

Streptococci ('Richards' strain; 30 mg. dry weight), in the metabolic mixture above (80 ml., but containing no glutamine), were allowed to glycolyze for 11 hr.; this required the addition of a further 8 ml. M/2$NaHCO_3$. The mixture was halved and *l(+)*-glutamic acid (578 μmol.) added to one half. Na_2CO_3 (0·5 g.) was added to each half, the mixtures separated from bacteria by centrifugation, and mercuric acetate added until a yellow precipitate formed. This happened almost immediately in the control, but in that containing glutamic acid, only after a considerable white precipitate had separated. Ethanol (250 ml.) was added to each mixture and after 2 hr. the precipitates were collected, washed with 80% alcohol, suspended in water, and decomposed with H_2S. The solutions after removal of HgS were evaporated *in vacuo* to *c.* 1 ml., taken to pH 3 by NaOH, and ethanol added to 60% (v/v). Glutamic acid separated from the solution to which it had been added: yield, 503 μmol. (87%). None was obtained from the other.

RESULTS

(1) *Characterization of volatile base.* The base, produced in a ratio of just less than 1 mol. per mol. of glutamine decomposed (McIlwain, 1946) could be determined consistently by Parnas' method for NH_3. It has been shown to be NH_3, rather than other simple amines, by comparison of the apparent quantities of substances when estimated by acidimetry and by Nessler's reagent (cf. François, 1907) (Table 1). The quantities were in close agreement, in the case of the base derived from glutamine

either during glycolysis or by acid hydrolysis; they differed markedly in the case of substituted ammonias.

Table 1. *Characterization of the volatile base from glutamine*

Metabolism (occupying about 1 hr.) was carried out anaerobically in Warburg vessels of 15–20 ml., with the reaction mixture described in the text. Material neutralizing acid after distillation in steam at 30° in a Parnas apparatus is described as a volatile base.

Origin of base	(*a*) Volatile base determined by acidimetric titration (μmol.)	(*b*) NH_3 determined by Nessler's reagent (μmol.)	$b/a \times 100$
Aliquots ($\frac{3}{10}$) of mixture from glycolysis by 6·9 mg. 'Richards' strain streptococci for 45 min. in the presence of 50 μmol. glutamine	2·28, 2·28	2·22, 2·38	98, 104
Aliquots ($\frac{1}{2}$) of mixture from glycolysis by 4·3 mg. Group G streptococci for 100 min. in the presence of 30 μmol. glutamine	5·80	5·78	100
10^{-2} M glutamine heated for 2 hr. at 100° in phosphate buffer, pH 6	7·30	7·48	102
Methylamine hydrochloride	2·50	0·56	22
Methylamine hydrochloride	1·25	0·21	17
Methylamine hydrochloride	0·63	0·09	14
Ethylamine hydrochloride	20	0·05	0·3
Dimethylamine hydrochloride	20	0·05	0·3

(2) '*Amino*-N' *change during reaction.* The N_2 produced by HNO_2 from the reaction mixture (after removal of NH_3) fell during glycolysis to the extent shown in Table 2 (A, B). The loss amounts to 92 % of the loss in labile amide-NH_3, and 107 % of the gain in NH_3. Such values are consistent with the main products from glutamine being glutamic acid and NH_3. As, however, carbohydrate metabolism is proceeding much more quickly than glutamine breakdown (Table 2 indicates the ratio Q_{CO_2}/Q_{NH_3} to be about 10), and the two processes are interdependent, many other products are possible.

(3) *Change in yield of succinic acid after treatment with chloramine-T and acid hydrolysis.* Direct production of succinic acid by the present streptococci during glycolysis in the presence or absence of

Table 2. *Loss of 'amino-N' during glutamine metabolism*

Experiments were carried out as described in Table 1, but in vessels of about 100 ml. capacity. Reaction mixtures contained glutamine (4×10^{-3} M) except the final one, which contained no added glutamine but 72 μmol. of ammonium glutamate. The CO_2 recorded is that from bicarbonate, mainly by displacement by the lactic acid formed in glycolysis (McIlwain, 1946) and the values are given to indicate the extent of glycolysis during the glutamine metabolism.

Reaction mixture	Substance determined	μmol. Originally	μmol. Finally	μmol. Change	Metabolic quotients (μmol./mg. dry wt./hr.)
A	Labile amide-N	47·7	11·1	−36·6	−3·17
	NH_3	0	29·8	+29·8	+2·58
	'Amino-N'	103	72	−31	−2·70
	CO_2 from bicarbonate	—	—	263	+22·8
B	Labile amide-N	60·5	19·5	−41	−1·61
	NH_3	0	36·2	+36·2	+1·42
	'Amino-N'	129	89	−40	−1·57
	CO_2 from bicarbonate	—	—	449	+17·1
C	Labile amide-N	0	0	0	0
	NH_3	74·7	71·3	−3·4	−0·24
	'Amino-N'	74·3	70·0	−4·3	−0·31
	CO_2 from bicarbonate	—	—	289	+21·0

glutamine, was found to be very small (McIlwain, 1946). Succinic acid as determined in reaction mixtures after Cohen's (1939) treatment, increased during glycolysis in the presence of glutamine. The only interpretation of this result, which is consistent with the known behaviour of substances as recorded by Cohen (1939), Örström, Örström, Krebs & Eggleston (1939), and in the experimental part of the present paper, is the production of a large proportion of glutamic acid. The increase in succinic acid formation then follows from its production in greater yield from glutamic acid than from glutamine. Assuming (*a*) that the yield of succinic acid from glutamic acid is 100 % and from glutamine after metabolism is the same as that before metabolism; and (*b*) that glutamic acid and NH_3 are the only products from glutamine, then the quantity of glutamic acid apparently formed can be calculated from the data of Table 3. The accuracy of the result is limited by the variable yield of succinic acid from glutamine, which has been noted above. In A, Table 3, this yield is 56 %; of the final succinic acid, 56 % of 36 μmol., or 20·1 μmol., is then contributed by glutamine leaving 53·2–20·1, or 33 μmol., to be derived from glutamic acid. This constitutes 1·14 mol. per mol. of glutamine decomposed. In B, Table 3, the corresponding ratio is 0·85.

Table 3. *Increase in succinic acid obtainable by Cohen's (1939) procedure, following streptococcal metabolism of glutamine*

Reaction mixtures (containing 4×10^{-3}M glutamine) and metabolic methods were those described in Table 2. Reaction B refers to the same batch of organisms as Table 2, B.

Reaction mixture	Substance determined	μmol. Originally	μmol. Finally	μmol. Change	Metabolic quotients (μmol./mg. dry wt./hr.)
A	Labile amide-N	65	36	−29	−2·4
	NH_3	0·5	26	+25·5	+2·1
	Succinic acid	36·3	53·2	+16·9	—
	CO_2 from bicarbonate	—	—	269	+22·3
B	Labile amide-N	60·5	19·5	−41	−1·61
	NH_3	0	36·2	+36·2	+1·42
	Succinic acid	37·2	46·8	+9·6	—
	CO_2 from bicarbonate	—	—	449	+17·1

(4) *Isolation of glutamic acid.* Foreman's method was applied, as described in the experimental part, to a mixture resulting from the action of the 'Richards' strain streptococci (27 mg. dry wt.) on the normal metabolic mixture containing glutamine (250 μmol.). Of this, 195 μmol. were decomposed and 85 μmol. of glutamic acid was isolated; it had m.p. 206° and mixed m.p., with an authentic specimen of *l*(+)-glutamic acid (m.p. 211°), 210°.

A higher yield was obtained by taking the reaction with glutamine to completion and separating the product by Neuberg & Kerb's (1912) method. This was applied to a mixture resulting from the action of 'Richards' strain streptococci (51 mg. dry wt.) in the normal metabolic mixture containing 878 μmol. of glutamine. After 10 hr., analysis of aliquots showed 35 μmol. of glutamine to remain; after 11 hr. the product was separated as described above and yielded 735 μmol. of glutamic acid, m.p. 203°; mixed m.p. with an authentic specimen of the *l*(+) acid (m.p. 211°), 209°. It was once recrystallized from aqueous ethanol, and dried *in vacuo* over P_2O_5 at 100°; it then melted at 211° and yielded (Kjeldahl) NH_3, 6·6 μmol./mg. (calc. for $C_5H_9O_4N$, 6·8). The optical rotation in 3N-HCl was

$$[\alpha]_D^{22°} = +31° (\pm 0·5°),$$

which is that of the 'natural' isomeride (cf. Huffman & Borsook, 1932).

(5) *Secondary changes in glutamic acid and* NH_3 *after their formation.* The changes which have been recorded in glutamine, NH_3, and 'amino-N' are only roughly in stoichiometric proportions. To see to what extent this could be attributed to changes in glutamic acid and NH_3 after their formation, streptococci were caused to glycolyze in the presence of added ammonium glutamate (C, Table 2). The disappearance of a small proportion of both NH_3 and 'amino-N' was observed, but without the formation of glutamine. The changes are of such a magnitude as may explain the greater change which has been observed to take place, during the main reaction, in glutamine than in NH_3 and 'amino-N'.

(6) *Influence of possible* NH_3 *acceptors on* NH_3 *formation from glutamine.* Ammonia formation by streptococci from glutamine during glycolysis, was followed in the presence and absence of substances which might combine with the NH_3 (Table 4).

Table 4. *Influence of added substances on glutamine breakdown*

Reactions between glutamine, glucose, the 'Richards' strain streptococci and added substances were carried out anaerobically in the bicarbonate-saline; controls lacked the added substance.*

Added substance (molarity)		NH_3 produced (% of control)	Glutamine reacting (% of control)	CO_2 evolution (% of control)
Pyruvate	$(1·4 \times 10^{-2})$	109	103	100
Pyruvate	$(3·5 \times 10^{-3})$	105	103	102
α-Keto-glutarate	$(1·4 \times 10^{-2})$	106	100	97
α-Keto-glutarate	$(3·5 \times 10^{-3})$	103	100	97
α-Keto-glutarate	$(1·4 \times 10^{-2})$*	98	—	100
α-Keto-glutarate	$(1·4 \times 10^{-3})$*	96	—	102
Oxaloacetate	$(1·4 \times 10^{-2})$*	105	—	102
Oxaloacetate	$(1·4 \times 10^{-3})$*	100	—	102
Inosine triphosphate	$(6·7 \times 10^{-3})$	100	100	104
Inosine triphosphate	$(1·4 \times 10^{-4})$	103	100	110
Hypoxanthine	(5×10^{-3})	100	—	105
Uracil	$(7·5 \times 10^{-3})$	51	45	86
Uracil	(5×10^{-3})	74	75	93

* The streptococci used were grown with added pyridoxin (see text).

Pyruvate, α-ketoglutarate, oxaloacetate, inosine triphosphate (Kleinzeller, 1942) and hypoxanthine were without marked effect. Uracil decreased NH_3 formation but also decreased glutamine breakdown and glycolysis. The growth of the streptococci in the present medium was sometimes slightly accelerated by pyridoxin, derivatives of which act as co-enzymes in decarboxylation of (Gale & Epps, 1944; Gunsalus, Bellamy & Umbreit, 1944; Baddily & Gale, 1945) and transamination (Lichstein, Gunsalus & Umbreit, 1945) between amino-acids. A batch of streptococci grown with 2×10^{-4}M pyridoxin reacted with glutamine at the usual rate and did not behave differently towards the keto-acids (Table 4).

DISCUSSION

The isolation of glutamic acid and characterization of NH_3 in the products from the action of non-proliferating streptococci on glutamine leave no doubt that the overall reaction is, to the extent of at least *c.* 85 %, a simple hydrolysis:

$$l(+)C_5H_{10}O_3N_2 + H_2O \rightarrow l(+)C_5H_9O_4N + NH_3.$$

The various determinations which were carried out during the course of the reaction did not indicate the temporary accumulation of other substances. Ammonia as produced from glutamine did not appear more capable of undergoing further changes in streptococci than did NH_3 added as such.

The following aspects of the relationship between glutamine and streptococci must be considered in assessing the present results.

(1) *Glutamine and glutamic acid as cell constituents.* A major need in growing organisms is the production of new bacterial substance. Pollack & Lindner (1942) calculated that 1 mg. of several organisms which needed added glutamine or glutamic acid, both contained and required in growth about 0·05 mg. (or 0·3 μmol.) of the substances. Taking 15 min. as a minimum mean generation time for the present streptococci, in this time 1 mg. of organisms of $Q_{glut.} - 1$ to -3 will have decomposed 0·25 to 0·75 μmol. of glutamine. They thus perform the reaction at about the rate at which they would be incorporating glutamine or glutamic acid into new cells, were they growing. This may be a coincidence or may indicate an association of the present reaction with growth. Gale (1946) observed that neither glutamic acid nor glutamine entered the cells of certain streptococci in the absence of substances which could act as energy-sources; this is relevant also to (3), below.

(2) *The stimulation of glycolysis by glutamine* nevertheless is not secondary to growth in the ordinary sense, as it occurs within two minutes of the addition of glutamine to glycolyzing streptococci. A function of glutamine in intermediary metabolism, such as that discussed earlier (McIlwain, 1946) remains likely, especially in view of many of the widespread biological activities of glutamine referred to by Archibald (1945*b*).

(3) *Dependence of glutamine breakdown on glycolysis.* If the computations of (1), above, reflect a functional connexion, it is one between glutamine breakdown and synthetic processes, which in very general terms might be expected to require the energy and materials made available by glycolysis. The means by which the connexion is normally effected, and by which a certain connexion is maintained in non-proliferating suspensions, remain unknown.

SUMMARY

1. Non-proliferating suspensions of haemolytic streptococci can convert $l(+)$-glutamine to $l(+)$-glutamic acid and NH_3.

2. The action differs from that of other 'glutaminases' in that it has so far been observed only during glycolysis; but intermediate compounds which might link glutamine decomposition with glycolysis have not been detected, nor have conditions been discovered under which the NH_3 takes part in secondary reactions at a comparable speed.

3. The rate of glutamine decomposition (1–3 μmol./mg. dry wt./hr.) is of the same order of magnitude as that at which glutamine or glutamic acid would be assimilated by growing organisms.

I am greatly indebted to Mr D. E. Hughes for assistance during these investigations, and to Dr J. H. Hale for advice on the preservation of streptococcal cultures.

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The Thyrotrophic Hormone, Thyroxine and Ascorbic Acid in Relationship to the Liver Glycogen of Guinea-pigs

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(*Received* 10 *September* 1945)

Accompanying the increase in the rate of metabolism, as induced experimentally by the administration of the thyrotrophic hormone or thyroxine, is a change in the ascorbic acid reserves of the body. A reduction in the ascorbic acid content of the livers and adrenals of experimental animals has been shown by Demole & Ippen (1935), Mosonyi (1936), Thaddea & Runne (1938), Sure & Theis (1939) and Farmer & Fribourg (1942), to follow injections of thyroxine, whilst Thaddea & Runne (1938) noted a similar result with the thyrotrophic hormone. With the pituitary hormone Loeser & Trikojus (1937) observed a fall in the ascorbic acid content of the adrenals of guinea-pigs whilst the liver ascorbic acid was unchanged or slightly elevated. They also found that by treatment for several weeks the vitamin content of the adrenals rose significantly above the normal value (a result confirmed in more extensive experiments by Trikojus & Ellis (1939)) whilst the content of the liver was normal. Paal & Brecht (1937) obtained different results in that thyroxine after 12 days brought about an increase in the adrenal ascorbic acid (rats and guinea pigs) while large doses of the thyrotrophic hormone (100 Junkmann-Schoeller units/day) led to subnormal values in the adrenal glands of guinea-pigs but did not affect the vitamin concentration in the livers of these animals.

The mobilization of liver glycogen produced by injections of the thyroid or pituitary hormone has been well established (cf. Harington, 1933; Eitel & Loeser, 1932; Eitel, Löhr & Loeser, 1933; Holden, 1934). According to Loeser (1936) the liver glycogen values reach a minimum after 7–9 days' treatment with thyroid hormone. Steffen & Zois (1938), while noting in 12-day experiments a marked fall in the liver glycogen of thyroxine-treated rats, were able to obtain a significant fall in the muscle but not in the liver glycogen of guinea-pigs treated with the thyrotrophic hormone (40 units/day).

That ascorbic acid promotes the storage of glycogen has been suggested by the work of Morelli & d'Ambrosio (1938), and other investigators. Furthermore, Fischbach & Terbrüggen (1938), using a small number of animals, claimed, from histological examinations, that the livers of guinea-pigs receiving large doses of thyrotrophic hormone together with ascorbic acid, contained abundant glycogen compared with the livers of animals receiving hormone alone, although both groups exhibited a comparable degree of thyroid hyperplasia. However, no effect on the part of ascorbic acid could be observed by these workers in the livers of thyroxine-treated animals. On the other hand, Thaddea & Scharsach (1940) found that administration of the vitamin tended to cause a return to normal of the liver glycogen and muscle lactic acid of guinea-pigs receiving either the pituitary or thyroid hormone. Hirsch (1936) and Steffen & Zois (1938) have reported the influence of large doses of ascorbic acid in partially preventing the fall in liver glycogen produced in guinea-pigs or in rats, respectively, by thyroxine. The experimental evidence is thus conflicting. Since it is generally accepted that the action of the thyrotrophic hormone is mediated by the thyroid gland, and that the consequent thyroid hyperplasia is associated with an additional release of thyroid hormone into the circulation, it would be expected that whatever the nature of the influence of ascorbic acid administered in large doses concurrently with thyroxine or the pituitary hormone, the effects would be in the same direction in both cases.

In the present investigation determinations have been made of the glycogen and ascorbic acid contents of the livers of guinea-pigs maintained on a standard scorbutic diet, with varying supplements of ascorbic acid, and in receipt of injections of thyroxine or thyrotrophic hormone.

EXPERIMENTAL

Immature guinea-pigs of both sexes and from the same stock (those from another stock were used in Exp. 1) were caged in groups not exceeding five animals and given *ad libitum* the scorbutic diet devised by Demole (1934) for 3–4 days prior to the beginning of each experiment. The animals in group III (Exp. 1) and group V (Exp. 2A) received a supplementary daily ration of fresh lucerne. Administration was then commenced daily for 12 days of minimal (0·5–1·0 mg.) or massive doses of ascorbic acid (Redoxon—Roche Products Ltd.) orally by pipette, or sodium ascorbate (Redoxon Forte—Roche Products Ltd.

Table 1. *Effects of administration of thyrotrophic hormone or thyroxine to guinea-pigs with or without large supplements of ascorbic acid*

Summary of Exps. 1–4 with averaged data for each group of animals

Exp. no.	Group no. and treatment (12 days)	No. of animals	Initial weight (g.)	Final weight (g.)	Thyroids (mg./100g. body wt.)	Adrenals (mg./100g. body wt.)	Liver: Glycogen (g./100 g.)	Liver: Ascorbic acid (mg./g.)	Thyroid histology
1	I. S.D. + 0·5 mg. A.A. (*per os*) daily	5	208	263	11·5	36·8	2·85	—	Normal
	II. S.D. + 0·5 mg. A.A. (*per os*) + 20 units T.H. daily	5	235	250	33·6	48·7	0·39	—	Intense activity; colloid pale and scanty
	III. S.D. + fresh lucerne + 100 mg. A.A. (*per os*) + 20 units T.H. daily	5	223	245	41·3	56·6	0·03	—	Intense activity; colloid pale and scanty to moderate
2A	I. S.D. + 200 mg. A.A. (*per os*) + 20 units T.H. daily	5	202	220	45·2	75·4	1·15	0·31	Intense activity; colloid scanty
	II. S.D. + 0·5 mg. A.A. (*per os*) + 20 units T.H. daily	5	203	212	45·7	66·5	1·02	0·07	As group I
	III. S.D. + 200 mg. A.A. (*per os*) daily	3	187	254	9·9	37·4	3·82	0·18	Normal
	IV. S.D. + 0·5 mg. A.A. (*per os*) daily	4	202	259	10·9	41·8	3·85	0·065	Normal
	V. S.D. + fresh lucerne + 100 mg. A.A. (*per os*) daily	4	193	273	10·3	38·9	4·34	0·24	Normal
2B	I. S.D. + 125 mg. S.A. (I.P.) + 20 units T.H. daily	4	215	222	47·1	64·5	1·47	0·34	Intense activity as group I, Exp. 2A
	II. S.D. + 125 mg. S.A. (I.P.) daily	4	205	263	9·9	39·0	3·78	0·21	Normal
3	I. S.D. + 125 mg. S.A. (I.P.) + 100 μg. T. (H.I.) daily	4	193	207	8·7	62·8	0·99	0·31	Normal (low epithelium)
	II. S.D. + 1 mg. A.A. (*per os*) + 100 μg. T. (H.I.) daily	4	197	206	9·0	57·9	1·20	0·08	Normal (low epithelium)
	III. S.D. + 125 mg. S.A. (I.P.) daily	4	193	229 (no fasting period)	9·7	44·9	4·03	0·24	Normal
4	I. S.D. + 125 mg. S.A. (I.P.) + 20 units T.H. daily	7	191	205	40·0	78·9	1·90	0·32	Intense activity; colloid scanty to moderate
	II. S.D. + 125 mg. S.A. (I.P.) daily	7	189	240	13·7	47·5	3·64	0·27	Normal
	III. S.D. + 0·5 mg. A.A. (*per os*) + 20 units T.H. daily	4	182	217	43·9	65·2	1·74	0·05	Intense activity; colloid scanty

S.D. = scorbutic diet; A.A. = ascorbic acid; S.A. = sodium ascorbate; T.H. = thyrotrophic hormone; T. = thyroxine; I.P. = intraperitoneally; H.I. = subcutaneously.

Table 2. *Effects of administration of thyrotrophic hormone or thyroxine to guinea-pigs with or without large supplements of ascorbic acid*

Exps. 1–4. Means of values obtained and their standard errors

Key to Table 2A	Treatment	Exp. no.	Group no.	No. in sample	Change in wt. (g./100 g. initial)		Thyroids (mg./100 g. body wt.)		Adrenals (mg./100 g.)		Liver glycogen (g./100 g. liver)		Liver ascorbic acid (mg./g. adrenal)		
				n	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	*n*	Mean	S.E.
a	S.D. + 0·5 mg. A.A.	1 2A	I IV	9	+27·51	1·21	11·27	0·44	39·04	1·66	3·291	0·532	4	0·065	0·001
b (i)	S.D. + 200 mg. A.A.	2A	III	3	+39·00	8·83	9·90	0·91	37·40	0·36	3·817	0·182	3	0·177	0·009
b (ii)	S.D. + 100 mg. A.A. and lucerne	2A	V	4	+44·13	7·20	10·28	0·34	38·93	3·48	4·343	0·856	4	0·240	0·022
b (iii)	S.D. + 125 mg. S.A.	2B 3	II III	8	+23·88	2·67	9·80	0·65	42·00	2·02	3·904	0·102	8	0·226	0·008
b	S.D. + excess A.A.			15	+32·30	3·62	9·95	0·38	40·26	1·43	4·003	0·271	15	0·220	0·007
A	No hormone			24	+30·50	2·33	10·44	0·31	39·80	1·07	3·736	0·265			
c	S.D. + 0·5 mg. A.A. + 20 units T.H.	1 2A	II II	10	+5·89	1·74	39·68	2·59	57·66	4·73	0·735	0·243	5	0·070	0·003
d (i)	S.D. + 200 mg. A.A. + 20 units T.H.	2A	I	5	+9·84	4·52	45·16	3·47	75·42	7·28	1·152	0·492	5	0·306	0·011
d (ii)	S.D. + 100 mg. A.A. + lucerne + 20 units T.H.	1	III	5	+10·38	2·72	41·26	3·12	56·56	6·52	0·028	0·028			
d (iii)	S.D. + 125 mg. S.A. + 20 units T.H.	2B	I	4	+3·20	2·55	47·15	2·36	64·55	5·08	1·468	0·446	4	0·338	0·025
d	S.D. + excess A.A. + 20 units T.H.			14	+8·14	2·06	44·34	1·79	65·58	4·14	0·841	0·263	9	0·320	0·013
B	Thyrotrophic hormone			24	+7·20	1·39	42·40	1·54	62·28	3·15	0·797	0·180			
e	S.D. + 1 mg. S.A. + 100 μg. T.	3	II	4	+4·63	3·62	8·98	0·74	57·88	5·40	1·200	0·338	4	0·079	0·002
f	S.D. + 125 mg. S.A. + 100 μg. T.	3	I	4	+7·00	1·05	8·70	0·31	62·85	5·36	0·990	0·156	4	0·315	0·009
C	Thyroxine			8	+5·81	1·80	8·84	0·38	60·36	3·65	1·095	0·177			
	Without fasting period														
g	S.D. + 125 mg. S.A.	4	II	7	+28·60	1·30	13·73	0·70	47·49	2·43	3·640	0·593	7	0·269	0·016
h	S.D. + 125 mg. S.A. + 20 units T.H.	4	I	7	+7·99	3·83	39·99	2·63	78·93	2·05	1·901	0·376	7	0·320	0·013
i	S.D. + 0·5 mg. A.A. + 20 units T.H.	4	III	4	+19·10	3·73	43·93	3·35	65·25	6·23	1·743	0·603	4	0·049	0·002

S.D. = scorbutic diet. A.A. = ascorbic acid. S.A. = sodium ascorbate. T.H. = thyrotrophic hormone. T. = thyroxine.

Table 2A. *Significant differences (from data in Table 2)*

Effects of treatments	ν	Diff.	S.E.	t	P
Change in weight					
Hormone effects:					
$B-A$	46	−23·30	2·71	8·59	<0·001
$C-A$	30	−24·69	2·94	5·89	<0·001
$B-C$	30	—	—	—	—
Ascorbic acid effect:					
$(d+f)-(c+e)$	20	—	—	—	—
$b-a$	17	—	—	—	—
Fasting effects (fasted):					
$d-b$	27*	−24·16	4·16	5·69	<0·001
(not fasted):					
$h-g$	12	−20·61	4·05	5·09	<0·001
Thyroid weights					
Hormone effects:					
$B-A$	46	+31·96	1·58	20·29	<0·001
$C-A$	30	—	—	—	—
$B-C$	30	+33·56	1·59	12·38	<0·001
Ascorbic acid effect:					
$(d+f)-(c+e)$	20	—	—	—	—
$b-a$	17	—	—	—	—
Fasting effects (fasted):					
$d-b$	27*	+34·39	1·83	6·12	<0·001
(not fasted):					
$h-g$	12	+26·26	2·72	9·66	<0·001
Adrenal weights					
Hormone effects:					
$B-A$	46	+22·48	3·33	6·75	<0·001
$C-A$	30	+20·56	3·80	7·42	<0·001
$B-C$	30	—	—	—	—
Ascorbic acid effect:					
$(d+f)-(c+e)$	20	—	—	—	—
$b-a$	17	—	—	—	—
Fasting effects (fasted):					
$d-b$	27*	+25·32	4·38	5·95	<0·001
(not fasted):					
$h-g$	12	+31·44	6·93	4·54	<0·001

Table 2A (*cont.*)

Effects of treatments	ν	Diff.	S.E.	t	P
Liver glycogen					
Hormone effects:					
$B-A$	46	−2·939	0·320	9·17	<0·001
$C-A$	30	−2·641	0·318	5·57	<0·001
$B-C$	30	—	—	—	—
Ascorbic acid effect:					
$(d+f)-(c+e)$	20	—	—	—	—
$b-a$	17	—	—	—	—
Fasting effects (fasted):					
$d-b$	27*	−3·268	0·378	8·63	<0·001
(not fasted):					
$h-g$	12	−1·739	0·702	2·48	<0·05
Liver ascorbic acid					
Hormone effects:					
$B-A$	46	—	—	—	—
$C-A$	30	—	—	—	—
$B-C$	30	—	—	—	—
Ascorbic acid effect:					
$(d+f)-(c+e)$	20	+0·244	0·009	8·01	<0·001
$b-a$	17	+0·155	0·008	10·40	<0·001
Fasting effects (fasted):					
$d-b$	27*	+0·100	0·011	7·19	<0·001
(not fasted):					
$h-g$	12	+0·051	0·021	2·43	<0·05

* For liver ascorbic acid, $\nu=22$.

or Cantan Forte—Bayer Pharma Ltd., Sydney) intraperitoneally, with or without injections of thyrotrophic hormone (Exps. 1, 2A, 2B, 4) or thyroxine (Exp. 3). The preparation of thyrotrophic hormone (Schering A.G.—batch 135H) was kindly presented by Prof. Schoeller and contained 10 Junkmann-Schoeller guinea-pig units/mg. and was stated to be free from growth, gonadotrophic and lactogenic hormones. The thyrotrophic hormone was dissolved in saline and injected intraperitoneally; the thyroxine was neutralized with alkaline saline and injected subcutaneously. Injections and weighings were made at the same time on each morning. Twice during the experimental periods each animal received cod-liver oil (0·2 ml.) by pipette.

On the evening of the last day of injections in Exps. 1–3 the animals were starved for 6 hr. (water being available) and then given aqueous fructose (50% (w/v); 0·75 g. fructose/100 g. body weight) by pipette at intervals to allow exactly 8 hr. between the time of administration and the despatch of each animal. In Exp. 4 the conditions were varied by allowing the animals access to food up to the time of their despatch on the morning following that of the last

injections. Fructose was not administered to the animals in these groups.

The animals were killed by a sharp blow on the neck and the livers immediately removed, a weighed portion treated for the determination of ascorbic acid, and the residue transferred to tared flasks containing aqueous potassium hydroxide (30% w/v) for glycogen assay. In Exp. 1 glycogen assays only were made. The analyses were carried out according to the method of Loeser & Trikojus (1937) for ascorbic acid (with Tillman's reagent) and by that of Eitel & Loeser (1932) for glycogen (sugar estimations according to Bertrand). Thyroid and adrenal weights were also recorded, and the thyroid glands were fixed in 10% formalin and examined histologically (eosin-haematoxylin sections).

RESULTS

Details of the several experiments and treatments, together with the averaged data for each group of animals, are presented in summarized form in Table 1.

The results (Table 1) were treated statistically. Table 2 shows the means, and their standard errors, of the values obtained in the several experiments, both for individual treatments and for the larger, combined samples of those animals receiving minimal and massive doses of ascorbic acid with and without hormone (thyrotrophic hormone or thyroxine). Fisher's *t*- and *z*-tests were used to test the significance of differences between the means and variances of any two samples, the criterion of significance being that the calculated values of t or z should be greater than the tabulated values corresponding to $P = 0{\cdot}01$ for the requisite number of degrees of freedom (ν).

The significant differences, set out in Table 2A, permit the following conclusions to be drawn.

Liver glycogen. (*a*) The liver glycogen of guinea-pigs is decreased by the administration of the thyrotrophic hormone or thyroxine, neither substance in the doses used being more effective than the other. (*b*) The decrease is similar when large doses of ascorbic acid (by injection or orally, with or without a supplement of fresh greens in the diet) are given in conjunction with either the pituitary hormone or thyroxine. (*c*) The decrease is not so marked in animals which had no fasting period before despatch as in those which had been fasted.

Liver ascorbic acid. (*a*) The concentration of ascorbic acid in the liver is increased by the administration of the thyrotrophic hormone or thyroxine when large doses of the vitamin are given together with the hormone. (*b*) The increase is not so marked in animals with no fasting period just prior to the conclusion of the experiment.

Body weight, etc. Large doses of ascorbic acid are without significant influence on the reduced body weight, on the thyroid and adrenal hypertrophy and on the modified picture of the thyroid gland occasioned by the injections of the thyrotrophic hormone (this last conclusion is not statistically significant, as quantitative measurements were not made during the microscopic examinations). The same is also true for thyroxine in regard to body weight and adrenal hypertrophy.

These findings are not inconsistent with the results of clinical studies on thyrotoxic patients (Eitel, 1938; Lewis, 1938; Lambie, 1939). In such cases the increase in the amount of the vitamin needed to ensure saturation is probably a secondary manifestation of the condition, since the administration of large doses is without influence on the basal metabolic rate. Logaras & Drummond (1938) found, in contrast to other workers, that thyroxine-treated rats receiving massive doses of vitamin A showed a greater tendency towards storage of the vitamin than control animals. These authors also point out that in clinical hyperthyroidism the liver reserves are usually abnormally high.

SUMMARY

1. The effects in the guinea-pig of administration of thyrotrophic hormone or of thyroxine have been shown to be uninfluenced by large doses of ascorbic acid, as judged by liver glycogen values, thyroid, adrenal and body weights and by histological examination of the thyroid glands.

2. The livers of guinea-pigs receiving injections of the thyrotrophic hormone or thyroxine have been shown to possess a significantly elevated capacity to concentrate ascorbic acid when large supplements of the vitamin are provided.

This investigation was carried out during the tenure by one of us (M. G. C.) of a scholarship as portion of a grant for thyroid research from the Australian National Health and Medical Research Council. We desire to acknowledge the help given by Miss N. McArthur, Haley Research Scholar, Walter and Eliza Hall Institute, in the statistical treatment of the results and by Mr W. J. Ellis during the experiments.

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The Basic Amino-acid Content of Proteins

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(*Received* 1 *April* 1946)

Whilst determinations of the basic amino-acids of proteins by the new enzymic, isotope dilution and microbiological procedures are tending towards satisfactory agreement, the literature of previous years reveals the considerable disparity in results obtained by what were until recently the standard methods of base-analysis. These can be classed under two main heads, direct or isolation methods which are generally modifications of the original Kossel procedure, and indirect methods usually involving colorimetry or manometry. As a rule, lower results have been obtained by direct than by indirect methods; this inconsistency is inherent in the methods themselves. The direct will tend to give a minimum figure since it requires a quantitative isolation of pure material which may be subject to unrecognized or underestimated losses. The indirect, on the other hand, may easily give a high figure, since working losses will generally be small and the method may be credited with a degree of specificity not warranted by the conditions obtaining in the solution under examination. Nevertheless, under ideal conditions, as for example in a clean solution of the bases, there is no reason why results of analysis by the two methods should not agree within the limits of error of the individual methods; the chief difficulty is that a clean and at the same time quantitative base-solution is not readily obtainable by the usual precipitation methods.

The analyst employing one particular approach to the problem will tend to treat with some reserve the results obtained by another worker employing a fundamentally different procedure, unless of course there is a considerable measure of agreement. As a corollary, it is only on very rare occasions that results obtained by one method have been tested by the application of another method to the same fraction. Such an approach would seem very desirable and in this paper an attempt has been made to present analytical results, obtained by methods developed in this laboratory, which have been borne out by independent analyses, using alternative methods, on the same base-solution. Before this scheme can be of any real value, however, three conditions must be fulfilled: (1) the protein from which the base-fraction is prepared must be of high purity, (2) the fraction should contain the basic amino-acids quantitatively and (3) should not contain any other substance which will subsequently interfere with one or other of the analytical methods employed.

Electrodialysis of a protein hydrolysate has been selected as the means which will most readily yield a quantitative return of the bases free from other protein constituents. The method has been rigorously examined as a quantitative procedure and it has been satisfactorily demonstrated that conditions (2) and (3) are adequately fulfilled. Solubility corrections, which are often somewhat arbitrary and may assume very significant proportions when the amount of material is small, are thereby eliminated. The fulfilment of condition (3) eliminates the identification of possible interfering factors and is an essential in the present scheme where lysine is determined in the first place on a difference-N basis.

The principle of the method of electrodialysis is well known. The basic amino-acids, placed in the centre compartment of a three-compartment cell and subjected to the influence of a direct current at a suitable pH, will carry a net positive charge and migrate to the cathode. By selecting a pH at which both the basic and the monoamino-monocarboxylic amino-acids are completely or substantially dissociated (i.e. when the net charge is maximal for the former, and zero for the latter) an almost complete separation is effected. Dicarboxylic

acids will migrate to the anode. This is the principle involved in the separation of the bases originally described by Foster & Schmidt (1921–2) and first employed as a quantitative method by Kuhn & Desnuelle (1937). The method has been applied to the analysis of a number of proteins by Albanese (1940), Theorell & Åkesson (1942) and others. Separation is not immediately complete, however, since some of the monoamino-acids enter the cathode compartment by diffusion, and represent a comparatively small, though significant proportion of the amount present in the centre compartment; this contamination is almost negligible after the cathode solution (subsequently referred to as the 'catholyte') is submitted to a second electrodialysis. To make quite certain that only basic material will be present in the solution to be used for analysis, a third run at the selected pH has invariably been made.

It is appropriate at this stage to examine the procedures of Albanese and of Theorell since they are similar to that employed in the present paper. Albanese's contention that all non-basic material is eliminated by a single run at the selected pH is not borne out by the later work of Gordon, Martin & Synge (1941) nor by the results described here, in which a significant amount of non-basic material is shown to be present in the catholyte at this stage. His subsequent procedure, using the NH_3-free catholyte, was to remove arginine as flavianate, then, after electrodialyzing away the excess flavianic acid, to separate histidine as the Hg complex, and to determine lysine by estimating the N in the remaining solution. This procedure is open to error since (*a*) the arginine flavianate is not recrystallized and will almost certainly contain some histidine and lysine, (*b*) if the catholyte is not free from monoamino-acids the histidine-Hg complex will contain other amino-acid-N, e.g. tyrosine, and (*c*) lysine is determined by N-difference and will be subject to interference from any extraneous N which has not been removed with the arginine and histidine precipitates. The secondary run for the purpose of removing flavianic acid will probably tend to reduce the extraneous N present. Theorell & Åkesson (1942), who employ an apparatus designed to minimize diffusion, carry out their analyses on a solution which also has been subjected to only one run at a controlled pH. They do not claim complete removal of monoamino-N from the catholyte in all cases and indeed some of their experiments indicate the presence in the catholyte of extraneous N equivalent to anything up to 5 % of the monoamino-N originally present. They make allowance for this by a correction in the N-distribution, a not altogether satisfactory procedure if an accurate lysine figure is desired. Since the extraneous N will normally include tyrosine-N, the colorimetric histidine value may be erroneous. Martin & Synge (1945), in their recent review on analytical methods, suggest means whereby the diffusion might be eliminated and the separation effected by a single run at a suitable pH, but it is doubtful whether the somewhat greater practical difficulties involved would be justified in view of the simplicity of returning the catholyte to the cell for re-dialysis as in the present arrangement.

In the present work, having obtained the catholyte free from non-basic material, it was then examined in the following way: NH_3 was removed, total N determined by a micro-Kjeldahl method, arginine and histidine by colorimetric methods, and lysine calculated by difference. The arginine content was also determined by hydrolysis with alkali, histidine was checked in two cases by a modified Kossel-Block procedure by an independent observer, and the majority of the lysine as well as a few of the arginine and histidine figures have been determined independently by enzymic methods. Hydroxylysine estimations have also been carried out in most cases, employing a periodate oxidation method.

METHODS

(1) *Hydrolysis of protein*

The protein is equilibrated under atmospheric conditions and a weighed amount put into a suitable round-bottomed flask fitted with a ground-in condenser. Concentrated A.R. HCl (36% w/w) is added (*c.* 20 ml./g. protein), the protein brought completely into solution in a water-bath at 35–40°, then sufficient hot distilled water added to bring the concentration of HCl to 20% (w/w). A glass boiling-rod is introduced and the solution boiled gently under reflux for 24 hr. The upper sides of the flask are protected from direct heat by standing on an asbestos plate with a small hole under the flask. The hydrolysate is then concentrated *in vacuo* several times to remove excess HCl and diluted to a suitable volume. A portion of this solution is removed for total N determination by the micro-Kjeldahl procedure described by Chibnall, Rees & Williams (1943*a*), and the remainder is used for electrodialysis.

Weighed amounts of the protein are simultaneously withdrawn for the determination of moisture and ash as described by Chibnall *et al.* (1943*a*).

(2) *Electrodialysis*

Description of apparatus. This is very similar to that described by Albanese (1940). The cell, however, is made from ebonite which requires no treatment to render it impervious to the solutions employed, and contiguous surfaces can be accurately machined so that no gaskets are required, and only a light film of grease is necessary. Such a cell has been in almost continuous use for over 5 years and shows no sign of deterioration. Recently a similar cell has been constructed from 'Perspex' by Mr E. F. Williams of this laboratory. This appears to be equally satisfactory and has the advantage of being transparent. The end compartments, of about 100 ml. capacity, are fitted with draining tubes.

The centre compartment has been constructed to contain about 150 ml. so that catholyte and washings may be transferred for re-electrodialysis without concentration; no draining tube is fitted so that the contents may be in constant circulation. The cathode membrane is vegetable parchment and the anode membrane formolized gelatin on a cloth base. Electrodes are of thin Pt foil, area about 10 sq. cm. and a current of 0·5 amp. is employed at a potential of 220 V. The contents of the compartments are cooled and the centre one continuously stirred. A milliammeter is included in the circuit.

Procedure. The end compartments are three-quarters filled with distilled water, the current switched on, a sample of the protein hydrolysate placed in the centre compartment, levels adjusted with distilled water, and cooling water and stirrer turned on. The amount of hydrolysate taken will depend on the proportion of base-N in the protein and on the number of determinations to be carried out on the catholyte, but, in any case the sample should be as small as possible. The duration of the first run will depend on the quantity and nature of the amino-acids present and also on the amount of HCl remaining; the time for a sample representing 1 g. protein is generally 4–5 hr. The current eventually drops to about 30–40 ma., when the catholyte is run off and the wash-liquid added. During this run the contents of the anode compartment should be run off hourly and replaced by distilled water. The main purpose at this stage is to remove most of the colour and all the HCl, free and bound. In addition to the bases, the catholyte will contain a large proportion of the other amino-acids since the pH of the centre compartment is very low during most of the run.

A little bromocresol purple is now added to the catholyte which is then adjusted to pH 5·8 by the addition of dilute H_2SO_4. The cell is thoroughly washed and set up as for the first run, the catholyte being placed in the centre compartment. Towards the end of the second run, which will normally occupy less than 1 hr., the current drops sharply to 20–30 ma., when the catholyte is run off. Stirring is maintained throughout the run, but the pH is not adjusted since it does not fall below about 4·3 and at this point the net charge of the monoamino-acids is extremely small. The catholyte is then subjected to 2 further runs at an initial pH of 5·8. The final catholyte, which generally has a pH of about 10, is concentrated 2 or 3 times *in vacuo* on a water-bath to remove the NH_3 present. It is then brought to pH 5–6 with dilute H_2SO_4 before being made to volume.

(3) *The colorimetric estimation of arginine*

The method now employed is essentially the same as the modification of the Sakaguchi reaction previously described (Macpherson, 1942), but since the present procedure increases the reliability, it is given in detail below. The most important innovation is the inclusion of standards; over a short concentration range the calibration curve is practically linear, but it has been found that with variations in temperature, timing and in quality of reagents, the slope of the curve may vary considerably. This has recently been verified independently by Williams & Watson (1944). The proportion of urea has been reduced, although there is still sufficient to deal effectively with the excess hypobromite. Too great an amount of urea competes excessively for the hypobromite making duplication of colour development less reliable, and only one addition of hypobromite is now required. The blank is also very much less. The principle of adding urea previous to hypobromite has been retained; it is believed that this was first proposed by Dubnoff & Borsook (1941) in their methods for glycocyamine and arginine.

Arginine of known purity is essential in this method and as standard in the estimation by alkaline hydrolysis. It is best prepared as arginine monohydrochloride from recrystallized monoflavianate by the method of Brand & Sandberg (1932). Standard solutions should be made up frequently.

Procedure. A sample of the catholyte containing 0·03–0·15 mg. arginine is measured into a 25 ml. standard flask and diluted to 5 ml. Add 1 ml. 10% (w/v) KOH followed by 2 ml. 0·1% α-naphthol in 50% ethanol; mix and add 1 ml. 5% (w/v) urea. Mix thoroughly and add 2 ml. KOBr (2 g. Br_2 in 100 ml. 5% (w/v) KOH). The manner of addition of the KOBr is important; it should be blown in from a rapid-delivery pipette held above the surface of the liquid and the flask shaken vigorously immediately after addition. Make to volume with distilled water, mix and read against the blank after 15 min. A photoelectric colorimeter and an Ilford 604 (spectrum green) filter were employed in the measurement of intensity; this was expressed as an extinction coefficient and plotted against concentration.

General notes. (*a*) Standard and unknown should be developed at the same time; (*b*) standards should be respectively weaker and stronger than the unknown; (*c*) each flask should contain the same volume of liquid when the colour is developed; (*d*) the reagents should be delivered into the bulb of the flask; (*e*) a reagent should be added to each flask in turn before proceeding with the addition of the next reagent; (*f*) solutions for comparison must be perfectly clear and free from bubbles; and (*g*) a suitable colour filter must be employed otherwise duplication may not be good, particularly in the case of arginine.

Interference from other substances. Of the substances present in the catholyte from a protein hydrolysate, only arginine will give a strong Sakaguchi reaction. Ornithine, lysine, citrulline and glucosamine do not interfere. NH_3 gives rise to erratic results and should be removed. Histidine has been reported as having an inhibitory effect on the development of the arginine colour (Weber, 1930; Dubnoff, 1941; Brand & Kassell, 1942) complicated by a chromogenic effect (Dubnoff, 1941). This may be true under certain conditions, but in the present procedure histidine alone has a slight chromogenic effect and will increase the extinction coefficient given by arginine if present in sufficient quantity. The interference is a function of the ratio histidine/arginine, and not until this ratio reaches a value of 3 or more does the interference assume any significance. The great majority of proteins have a ratio considerably <1; notable exceptions are the globins of blood proteins where the ratio is >2 and insulin where it is >1. In all the proteins examined, therefore, the arginine value will be unaffected by the histidine present.

(4) *The estimation of arginine by alkaline hydrolysis*

On prolonged boiling with strong alkali, arginine yields one-half its N as NH_3. This is the principle of the Van Slyke (1911) method for the estimation of arginine which has been

further investigated by Plimmer (1916) and others. The method, however, has not been extensively employed, chiefly because of the interference of other amino-acids, notably cystine and the hydroxyamino-acids. This objection still holds for the base-fraction obtained by precipitation with phosphotungstic acid, since cystine is also partially precipitated and yields a considerable amount of NH_3 on alkaline hydrolysis. The correction factor is further complicated by the solubility of arginine phosphotungstate. The catholyte obtained by repeated electrodialysis of a protein hydrolysate as described above is, however, an eminently suitable medium for the application of the method, since there is no significant loss of arginine on dialysis and in the great majority of cases, when pure protein is employed, the solution will contain the three bases only. As will be shown, the interference from histidine and lysine is negligible and the only other substance likely to interfere is hydroxylysine, which has been found in small amount in only two of the proteins examined.

Accordingly, the method has been developed as a quantitative micro-method for the determination of arginine in proteins, primarily as a check on the values found by the more convenient colorimetric method. The original methods of Van Slyke and Plimmer have been modified in that (*a*) the basic amino-acids are separated by electrodialysis, (*b*) more concentrated alkali is used, (*c*) smaller quantities of arginine are required and (*d*) a modified apparatus is employed.

Description of apparatus. This apparatus is constructed wholly of Pyrex glass. The digestion-tube is thick-walled, approximately 9 in. long and 1·5 in. diameter with a B34 standard joint; the digestion-tube head carries the steam delivery tube and a reflux condenser, short and of wide bore to prevent 'pumping' of condensate whilst steam-distilling. A splash-head may be fitted to the top of the reflux and connected to the vertical distillation condenser which may be of narrower bore; the delivery end is tapered somewhat. Rubber connexions should be dispensed with where possible.

Procedure. Prepare an 80% (w/v) solution of A.R. KOH; the solution should be boiled for about an hour before adjusting the volume. It should not require filtration. The catholyte, or a portion of it, adjusted to about pH 5 with dilute H_2SO_4, should be concentrated if necessary so that 2 ml. contains 1–2 mg. arginine-N. Having steamed out the apparatus, put a 2 ml. sample of solution into the digestion-tube and introduce a small glass boiling-rod to prevent bumping. Lightly grease both the B34 joint and the tap to the steam supply with good quality N-free lubricant. Now place the receiver, containing the requisite quantity of N/70 HCl, in position with the tip of the delivery tube just below the surface of the acid. The receiver should be closed except for a soda-lime guard tube. Add 10 ml. 80% (w/v) KOH to the digestion-tube and immediately connect to the reflux, the tap to the steam supply being closed. The steam delivery tube should be of such a length that it just reaches, or is just above, the liquid in the digestion-tube. Water is run through the reflux condenser, and a small flame, sufficient to keep the solution boiling gently, is placed under the digestion-tube. It is recommended that the tube be supported on a piece of gauze placed over a 1 in. hole in an asbestos plate. Boiling, which proceeds smoothly, is continued for exactly 4 hr., when a vigorous current of steam is blown through the solution with the small burner still in position. Water is diverted from the reflux to the distillation condenser and distillation continued until about 75 ml. condensate have been collected. Titrate against N/70 NaOH.

(*Note.* Pyrex glass is rapidly attacked by strong alkali and the digestion-tubes as originally supplied will only withstand one, or at the most two, estimations. The tubes, however, should not be discarded for it will be found that on melting and remoulding the bottom of the tubes so that the pitting due to the alkali is removed, the glass will now be much more resistant to the alkali and can subsequently be employed for much longer periods.)

Recovery of arginine. The blank on the apparatus, with 2 ml. distilled water, 10 ml. 80% (w/v) KOH and after 4 hr. boiling, should not be more than 0·02 ml. N/70 HCl. Recovery of NH_3 from a 2 ml. sample of standard $(NH_4)_2SO_4$ is 100%±0·1.

Van Slyke (1911) states that recovery of arginine is quantitative under the conditions he employs. Plimmer (1916) finds somewhat erratic recoveries, usually less, but sometimes more than 100%; this may have been due, as he suggests, to impure arginine. Accordingly a series of experiments was run, under the exact conditions described above, on 2 ml. samples of solutions of pure arginine monohydrochloride, prepared as described under the colorimetric estimation. For digestion times of 0·5, 1, 2, 4 and 6·5 hr., the respective percentage recoveries of arginine-N, calculated as $2 \times NH_3$-N evolved, were 94·4, 95·0, 96·1, 97·0 and 97·8. Duplicates on these recoveries, from 1 hr. upwards, agreed within 0·2%. Whilst the recovery is gradually increasing, a much longer digestion period will obviously be required before it is quantitative in spite of the fact that the alkali employed is more concentrated than that of Van Slyke. When plotted against digestion time, the recovery values lie on a smooth curve which flattens out markedly from about 2 hr. upwards. Since recoveries have been shown to be consistent for any one digestion time, particularly in the flatter portion of the curve, it is justifiable to take the recovery at some arbitrary time in this range and to apply the appropriate correction. Thus for a 4 hr. digestion period, arginine-N = 1·03 ($2 \times NH_3$-N).

The incomplete recovery of arginine and the necessity for the drastic alkali treatment are probably due to the formation of citrulline, which is only slowly broken down by alkali. It has been stated by Hellerman & Stock (1938) and by Warner (1942) that citrulline is formed during the alkaline hydrolysis of arginine, part of the arginine yielding urea and ornithine, and part, NH_3 and citrulline. Warner also showed that subsequent breakdown of citrulline is slow; this was confirmed under the present conditions. After 1 and 4 hr. digestion, the NH_3-N recovered from citrulline was 28·03% and 30·45% of the total citrulline-N or 84·09% and 91·35% of the N available for NH_3 formation. Quantitative recovery of NH_3 from citrulline would therefore require prolonged digestion.

Interference from histidine and lysine. After 4 hr. hydrolysis under the above conditions, histidine yields NH_3 corresponding to 0·3% of the total histidine-N present, and lysine yields NH_3 equal to 0·17% of the total lysine-N present. Thus interference from these bases will be negligible except in an extreme case where they represent a very high proportion of the base-N of a protein.

(5) *The colorimetric estimation of histidine*

This method is carried out as previously described (Macpherson, 1942) with minor modifications. Standards

are now included and the quantity of ethanol has been reduced. A pure histidine sample was prepared by the method of Vickery (1942); a good specimen of the monohydrochloride monohydrate was obtained from Roche Products Ltd. Standards should be made up frequently.

Procedure. Measure a sample of the solution containing 0·02–0·10 mg. histidine into a 25 ml. volumetric flask and dilute to 5 ml. Add 1 ml. 1% sulphanilic acid in N-HCl and 1 ml. 5% (w/v) $NaNO_2$. Mix and stand 5 min., shaking the flask occasionally. Now blow in, from a rapid-delivery pipette, 3 ml. 20% (w/v) Na_2CO_3 (cryst.). Shake for about 10 sec. and add 10 ml. 20% (v/v) ethanol. Mix whilst cooling under the tap. Make to volume, mix and read in the photoelectric colorimeter using the same filter as for arginine.

Interference from other substances. The only likely serious interference would arise from the presence of tyrosine which also gives a strong colour with diazotized sulphanilic acid. Tyrosine, however, has been completely removed during the electrodialysis and the final catholyte should give no trace of colour with Lugg's (1937) very sensitive test for tyrosine. NH_3 has been removed and the other substances mentioned under arginine do not interfere. Histamine and substituted histidines such as carnosine and thiolhistidine would interfere but will not normally be present. Arginine does not interfere even in very great excess; base-solutions have been made up approximating the catholytes obtained from salmine and tobacco mosaic virus, where there are large amounts of arginine but no histidine, and no significant interference can be detected. The method may also be employed in the estimation of histamine and substituted histidines.

Independent estimations of histidine, arginine, lysine and hydroxylysine

(6) *Gravimetric estimation of histidine.* This was carried out by Mr E. F. Williams according to the method described by Chibnall *et al.* (1943*b*). In the present case the method is applied directly to the catholyte, so that all preliminary stages are eliminated, and the procedure consists essentially in removing the histidine as the Ag compound, subsequently breaking down and isolating as the nitranilate.

(7) *Estimation of hydroxylysine.* Hydroxylysine estimations were carried out by Mr M. W. Rees employing the periodate method of Van Slyke, Hiller & MacFadyen (1941) applied to the catholyte.

(8) *Enzymic estimations of arginine, histidine and lysine.* These were performed by Dr E. F. Gale, employing the specific decarboxylases according to his own recent methods (1945, 1946).

Description of proteins analyzed

N content as percentage dry ash-free protein

Casein. Prepared by Mr M. W. Rees by the method of Van Slyke and Baker (1918). Purified three times by the method of Cohn & Hendry (1923). N = 15·65%.

Gliadin. Prepared by Mr M. W. Rees by the method of Osborne & Mendel (1912). Precipitated three times. N = 17·66%.

Zein. Purified by Mr M. W. Rees. Commercial zein dissolved in ethanol and precipitated with an equal volume of ether; the procedure repeated three times in all. 'Hyflo super cel' added to resulting gel till dry enough to make press cakes. Cakes squeezed in hydraulic press and the syrup filtered through a tight filter pad. Zein precipitated by pouring slowly into dilute NaCl with vigorous stirring. Centrifuged, washed free from NaCl and air-dried at room temperature. N = 16·24%.

Collagen. Sample from Dr D. Jordan Lloyd. N = 18·2%.

Gelatin. Coignet 'Gold Label'. N = 18·0%.

Myosin. Prepared by Dr K. Bailey. Three times precipitated rabbit myosin (Bailey, 1942) dried in ethanol and ether. Extracted with M-KCl to reduce the small amount of nucleic acid-P (*c.* 0·05%) present in unextracted preparations and to remove traces of tropomyosin (Bailey, unpublished). Washed with water till free from Cl^-, dried in ethanol and ether. N = 16·8%.

Silk fibroin. Prepared by Dr K. Bailey. Cocoons boiled with water to remove sericin, the silk teased out and extracted successively with acetone, ethanol and ether. N = 18·7%.

Tobacco mosaic virus. Sample from Dr N. W. Pirie. Freed from nucleic acid by heat-coagulation in 0·1 N-NaCl, pH 6·5, for 2 min. at 100° the precipitated protein washed 4 times with water, and dried with ethanol and ether. N = 16·5%.

Wool keratin. Romney, New Zealand. Prepared by Dr K. Bailey according to the method described by him (Bailey, 1937). N = 16·3%.

Salmine. Commercial sample salmine sulphate purified by Dr G. R. Tristram by a procedure based on Kossel (1928). N = 24·7% calculated on the sulphate.

Edestin. N = 18·65; *insulin*, N = 15·54; *β-lactoglobulin*, N = 15·6; *ovalbumin*, N = 15·76%. Described by Chibnall *et al.* (1943*a*).

Haemoglobin. Horse CO-haemoglobin, prepared and recrystallized by Mr M. W. Rees according to the method of Adair & Adair (1934). Heat coagulated. N = 16·8%.

RESULTS

Recovery experiments on electrodialysis

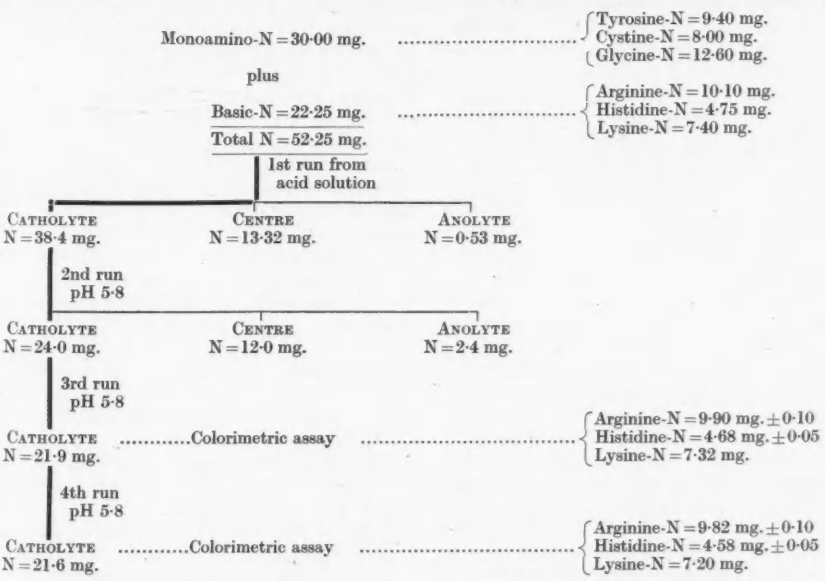
In all recovery experiments, the procedure adopted for a protein hydrolysate was followed, viz. an initial electrodialysis at acid pH in the presence of HCl, followed by three runs at an initial pH of 5·8, the final catholyte then being concentrated *in vacuo*, at its own (alkaline) pH before being diluted to volume at pH 5–6.

Exp. (*a*). Here arbitrary mixtures of the three bases only were electrodialyzed under the conditions above. Arginine and histidine were then determined colorimetrically and lysine estimated by N-difference. Over a series of experiments, the recovery of arginine was 98–100% and of histidine 97–99%, both involving a possible error in colorimetry of ± 1%. The lysine recovery averaged about 99%, but in this case the error may be greater since the lysine figure will carry the errors of three other estimations and the error will also depend on the proportion of lysine to the other bases.

Exp. (*b*). Another series of experiments was carried out in which complete amino-acid mixtures

approximately representing the composition of protein hydrolysates were subjected to electrodialysis. In some cases the mixture was boiled with 20% HCl as in the hydrolysis of a protein, and in others the electrodialysis was carried out directly. No significant destruction of the bases could be detected under these conditions as recoveries were equally satisfactory after the HCl treatment. Typical of the results obtained are the recoveries from a mixture representing insulin which had been boiled with HCl for 24 hr. then electrodialyzed in the usual way: arginine 98% by colorimetry, 99·5% by alkaline digestion; histidine 99·5% by colorimetry and lysine 98·8% by N-difference. The respective possible errors in the estimations are ±1, ±1, ±1, and ±3%.

Exp. (*c*). A third type of experiment was designed not only to test the base-recoveries, but also to assess the interference from non-basic material at various stages in the electrodialysis. An acid solution of the bases with the addition of tyrosine, cystine and glycine was subjected to the routine electrodialysis procedure, total N estimations made on the cathode and centre compartment solutions after the first two runs, and total N, arginine and histidine estimations on the catholytes from the third and fourth runs. The results are shown in the flow-sheet below.


Monoamino-N = 30·00 mg.
Tyrosine-N = 9·40 mg.
Cystine-N = 8·00 mg.
Glycine-N = 12·60 mg.
plus
Basic-N = 22·25 mg.
Arginine-N = 10·10 mg.
Histidine-N = 4·75 mg.
Lysine-N = 7·40 mg.
Total N = 52·25 mg.
1st run from acid solution
Catholyte N = 38·4 mg.
Centre N = 13·32 mg.
Anolyte N = 0·53 mg.
2nd run pH 5·8
Catholyte N = 24·0 mg.
Centre N = 12·0 mg.
Anolyte N = 2·4 mg.
3rd run pH 5·8
Catholyte N = 21·9 mg.
Colorimetric assay
Arginine-N = 9·90 mg. ±0·10
Histidine-N = 4·68 mg. ±0·05
Lysine-N = 7·32 mg.
4th run pH 5·8
Catholyte N = 21·6 mg.
Colorimetric assay
Arginine-N = 9·82 mg. ±0·10
Histidine-N = 4·58 mg. ±0·05
Lysine-N = 7·20 mg.

After the first run, the catholyte contains 16·15 mg., or nearly 54%, of the original monoamino-N and after a further run at an initial pH of 5·8 it contains 1·75 mg., or 5·8%, of the original monoamino-N. In the third catholyte no tyrosine or cystine could be detected and the recovery of total base-N is 98·4%; the individual recoveries are arginine 98%, histidine 98·5% and lysine 99%. In the fourth catholyte recoveries are arginine 97·3%, histidine 96·5% and lysine 97·3%. These recoveries are a little low, but this may be due to the cumulative working loss being somewhat greater than usual since this experiment involved four concentrations *in vacuo* and the removal of numerous samples.

Analyses of proteins

In Table 1 are collected the basic amino-acid contents of the proteins previously described.

DISCUSSION

The foregoing presents a simple scheme of base-analysis, economical of time and material, which when applied with care is capable of yielding results of considerable accuracy. The merit of the electrodialysis resides in the fact that a clean and quantitative separation of the bases is attained and the resulting catholyte is suitable for the application of any of the methods of base-analysis, whilst interference with a particular method can be readily assessed on account of the limited number of substances present. It provides, too, what is probably the most satisfactory approach to the detection of basic material other than arginine, histidine and lysine. The method can, if necessary, be employed with very small quantities of material; even where

Table 1. *Comparison of figures obtained by the application of independent methods to the catholytes*

(Values quoted are N as % protein-N.)

Protein	Arginine	Histidine	Lysine
Casein	8·4 C	5·5 C	10·25 N
	8·3 D		
	7·9* E		10·1* E
Collagen	15·2 C	1·1 C	4·7 N
	15·3 D		
Edestin	28·7 C	4·20 C	2·30 N
	27·8 D		
	27·8 E	4·13 E	2·40 E
Gelatin	15·3 C	1·1 C	4·9 N
Gliadin	4·7 C	3·2 C	1·2 N
	4·7 D		
Haemoglobin (horse)	7·2 C	13·5 C	9·8 N
	7·2 D		
Insulin	6·35 C	8·55 C	3·1 N
	6·20 D	8·5 G	3·0† G
	6·36 E		3·0 E
β-Lactoglobulin	5·8 C	2·85 C	13·6 N
	5·95 D		
	5·8 E		13·8 E
Myosin	14·2 C	3·9 C	13·6 N
	14·3 D		13·8 E
Ovalbumin	11·7 C	4·1 C	7·4 N
	11·7 D	4·0 G	7·45* E
		4·2 E	
Salmine	86·5 C	Nil	Nil N
Silk fibroin	1·90 C	0·53 C	0·70 N
	1·87 D		
‡Tobacco mosaic virus	20·5 C	Nil	1·2 N
Wool keratin	20·5 C	1·75 C	3·1 N
	19·9 D		3·25 E
Zein (commercial)	3·20 C	2·20 C	Nil N
	3·37 D		
Zein (purified)	3·25 C	2·10 C	Nil N

C = colorimetry, D = alkaline digestion, E = enzymic, G = gravimetric, N = N-difference.

* On hydrolysate.

† This case is discussed by Chibnall (1946).

‡ Freed from nucleic acid.

lysine is determined by N-difference, requiring a micro-Kjeldahl estimation, only a few mg. of base-N need be present and the dialysis time is then considerably shortened. Less than 0·5 mg. base-N has been electrodialyzed in the presence of the other amino-acids without loss, although in such cases a smaller apparatus would be desirable. As in all micro-analytical work, attention to detail is imperative and it is appropriate at this juncture to emphasize precautions which must be observed and to indicate some of the sources of error which have been encountered in perfecting the methods described.

With a properly constructed three-compartment cell electrodialysis should present no difficulty and working losses will be very small. Loss may be incurred, however, if contiguous faces of the cell sections do not meet accurately, since even with gaskets there may be some leakage between sections. Another possible source of loss is a perforated membrane, particularly the gelatin anode membrane, through which there may be some loss of material from the centre compartment whilst the anode compartment solution is being run off. Some such mechanical loss is probably responsible for the somewhat lower figures obtained in one or two earlier analyses, e.g. the haemoglobin figures quoted by Chibnall (1946). Fresh membranes should be inserted after each set of runs; they need not be replaced after every run, but where the hydrolysate contains much humin the cathode membrane becomes very dirty and clogged and should be renewed before proceeding with the three runs at pH 5·8. Of the bases, histidine would appear to be the most difficult to recover quantitatively in the catholyte. It is the least basic and at pH 5·8 dissociation, whilst substantial, is not complete; dissociation is complete when the pH has dropped to about 5 but, on the other hand, in the alkaline catholyte, histidine carries a net negative charge which would tend to return it to the centre compartment; this tendency in turn will be opposed by the acidity in that compartment. Presumably, therefore, some sort of equilibrium is reached at the membrane, across which there exists a considerable pH drop when the contents of the centre compartment are in rapid circulation. The recovery of histidine might be expected to be somewhat low, and the fact that it is almost as satisfactory as that of the other bases may be due in part to the unidirectional electroendosmotic flow from centre to cathode compartment. Stirring of the centre compartment should be continuous so as to avoid building up a local alkaline pH on the anode side of the cathode membrane.

Concentration *in vacuo* of the final catholyte must be thorough since the last traces of NH_3 are difficult to remove. Catholytes, like the standard base-solutions, do not keep well and should be analyzed as soon as possible. The use of preservatives is not recommended when colorimetric methods are to be employed and is generally out of the question for enzymic or microbiological methods. When it is desired to preserve the solutions for some time they should be made slightly acid with HCl and evaporated to dryness.

Colorimetric methods are often much maligned and regarded as giving at best only an approximate estimate of the amount of material present. This is due in large part to the fact that on account of their suitability for application to large numbers of routine determinations they have often been employed where a comparative figure is of as much

moment as an absolute one. Owing to their extreme sensitiveness and liability to interference they must be carried out under careful control when employed as analytical procedures, but when so applied they can equal any other method in simplicity and reliability. In the colorimetric methods described, inclusion of standards as a determinant of the slope of the calibration curve is essential. Many inconsistent and erroneous results were obtained before this was realized. Given reliable arginine and histidine standards, which must be freshly prepared, there should be no difficulty in obtaining reproducible figures provided also that the general notes on technique given under the arginine procedure are carefully observed. One point which has not been mentioned is that significant amounts of metals such as Fe, Cu, Ag, etc., should not be present since they will interfere with colour formation. The presence of the alkali metals is immaterial as far as the colorimetric methods are concerned, and the alkaline earths do not affect the estimations unless present in amount sufficient to give rise to a turbidity at the alkalinity at which the colours are developed.

One of the most likely sources of error in the scheme is the determination of total N by the micro-Kjeldahl method. Although a very accurate method when applied to completely digested protein or amino-acid, it is not always realized, as Chibnall *et al.* (1943*a*) have pointed out, how difficult the attainment of complete digestion may sometimes be. Of the bases, histidine, and lysine in particular are difficult to break down completely and require prolonged and vigorous oxidation in presence of a suitable catalyst. This becomes of increasing importance in the case of a catholyte where the proportions of histidine and lysine are much greater than in the protein or protein hydrolysate. When results are expressed, as they are in the present paper, as amino-acid-N as % of protein-N, then a low N on the protein hydrolysate will give rise to high figures for the amino-acids; a low N on the catholyte will mean a low lysine figure where this is calculated from N-difference. Where the lysine content of a protein is of the same order as the arginine and histidine, then it can be calculated from the N figures on the catholyte with an accuracy dependent on the errors cumulating from the total N, arginine-N and histidine-N determinations, that is within about 3%. In cases like edestin and tobacco mosaic virus where the lysine-N is a small percentage of the total N of the catholyte, then the error in the calculation may be very much greater and the method cannot be recommended as a means of determining lysine, except as a first approximation. Again, in an extreme case such as salmine, where the base-N is very high and appears to be all arginine-N, it is difficult to state with certainty whether lysine is present or not, and an independent determination, preferably by an enzymic or microbiological method, will be required. In a very large number of proteins, however, the lysine represents a sufficiently large proportion of the base-N to make its calculation from N-difference sufficiently accurate for analytical purposes.

It has been demonstrated that one run at a controlled pH, as has been employed by several workers, is not sufficient to free the catholyte completely from non-basic N, and this may give rise to various errors depending on the subsequent methods of analysis employed. At least two such runs, and preferably three, are recommended.

Table 1 shows the close agreement obtained by the application of independent methods to the catholytes. Whilst a greater number of gravimetric and enzymic analyses would have been desirable, this was not feasible because of the already heavy demand on the time of the workers concerned, but a sufficient number have been performed to provide a reasonable check on the methods proposed. The results also bear out the statement made earlier that given a clean solution of the bases, there is no reason why analyses by different methods should not agree within the limits of error of the individual methods.

Comparison of the present results and other published figures as shown in Tables 2–4 has already been discussed by Chibnall (1946) from whose paper the bulk of these tables has been taken. It is therefore unnecessary to do more than outline the conclusions to be drawn from this comparison. In the case of arginine, agreement among all the methods employed is fairly satisfactory, except in a protein like silk fibroin where the arginine content is small, and the recovery by isolation tends to suffer from solubility and other working losses. Agreement in the more recent histidine values is generally good, but most of the gravimetric figures are low; this is probably due to the difficulties attendant upon the separation and isolation of the small amount of histidine present in most proteins. Where the histidine content is large, as in haemoglobin, the discrepancy is not so marked. As Chibnall has pointed out, some histidine is apparently destroyed by the use of Cu_2O for the removal of cystine. It has been demonstrated in the course of this work that the addition of Cu_2O to a solution containing histidine, under the conditions for mercaptide formation, will result in a loss of up to 35% of the histidine when this is determined by colorimetric and gravimetric assay. It is believed that the histidine values of Vickery & Winternitz (1944), which are somewhat higher than most other gravimetric figures, are still low. The present figure for edestin, which is closely checked by an

Table 2. *Arginine content of proteins, showing comparison with other published values*

(Values quoted are N as % protein-N.)

	(*a*)	(*b*)	(*c*)	(*d*)	(*e*)	(*f*)	(*g*)
Casein	8·3	7·36	7·9	7·9[5]	7·7	8·0[20] 7·3[25]	—
Collagen	15·2	—	—	—	—	—	—
Edestin	28·7	26·3	—	28·85[6]	28·9 28·2[6]	—	—
Gelatin	15·3	11·6	—	—	15·3	16·6[11] 16·2[20] 14·6[25]	—
Gliadin	4·7	—	—	—	4·7	—	—
Haemoglobin (horse)	7·2	6·96	6·92	6·4[7]	6·92 7·2[6]	6·55[11]	7·1[12]
Insulin	6·35	—	—	6·37[8]	—	—	—
β-Lactoglobulin	5·9	—	5·82	5·95[6]	—	5·95[11] 5·76[20]	—
Myosin	14·2	—	—	13·4[28]	—	—	—
Ovalbumin	11·7	—	—	11·44[6]	11·7 11·7[6]	11·4[11] 12·1[20]	—
Salmine	86·5	—	—	90·2[27]	—	—	—
Silk fibroin	1·9	—	—	1·3[5]	1·3	1·7[11] 1·9[20]	—
Tobacco mosaic virus	20·5	—	—	18·2[22, 23]	—	—	—
Wool keratin	20·2	—	—	20·2[10]	20·2	—	—
Zein	3·3	—	—	3·2[9]	3·2	—	—

(*a*) Author, from electrodialysis, colorimetry and alkaline digestion.
(*b*) Albanese[1], from electrodialysis and flavianic acid precipitation.
(*c*) Gale[3], specific decarboxylase applied to hydrolysate.
(*d*) Modifications of the silver precipitation method.
(*e*) Vickery[4], flavianic acid method.
(*f*) Microbiological assay.
(*g*) Isotope dilution method.

Indices denote numbered references at the end of the paper.

Table 3. *Histidine content of proteins, showing comparison with other published values*

(Values quoted are N as % protein-N.)

	(*a*)	(*b*)	(*c*)	(*d*)	(*e*)	(*f*)	(*g*)
Casein	5·5	5·36	—	3·15[5]	4·35	—	4·85[20] 5·2[24] 4·5[25]
Collagen	1·1	—	—	—	—	—	—
Edestin	4·2	4·26	—	3·50[6]	3·65	—	—
Gelatin	1·1	1·14	—	—	0·76	—	0·87[20] 1·2[25]
Gliadin	3·2	—	3·5	—	2·75	—	—
Haemoglobin (horse)	13·5	14·48	12·5	12·4[7]	12·4	—	—
Insulin	8·55	—	—	—	—	—	—
β-Lactoglobulin	2·85	—	—	2·69[6]	—	2·75	2·60[20]
Myosin	3·9	—	—	2·8[28]	—	—	—
Ovalbumin	4·1	—	—	2·5[6]	—	—	3·96[20]
Salmine	Nil	—	—	—	—	—	—
Silk fibroin	0·53	—	—	0·10[16]	—	—	0·59[20] 0·49[21]
Tobacco mosaic virus	Nil	—	—	Nil[22]	—	—	—
Wool keratin	1·75	—	—	1·23[10]	—	—	—
Zein	2·2	—	—	—	1·43	—	—

(*a*) Author, from electrodialysis and colorimetry.
(*b*) Albanese[1], from electrodialysis and $HgCl_2$.
(*c*) Gale[2], specific decarboxylase applied to hydrolysate.
(*d*) Modifications of silver precipitation method.
(*e*) Vickery[13, 14], 3:4-dichlorobenzene-sulphonic acid method.
(*f*) Brand's[15] method.
(*g*) Microbiological assay.

Indices denote numbered references at the end of the paper.

Table 4. *Lysine content of proteins, showing comparison with other published values*

(Values quoted are N as % protein-N.)

	(*a*)	(*b*)	(*c*)	(*d*)	(*e*)	(*f*)
Casein	10·2	10·22	10·1	7·7[5]	10·2[17] 9·43[20] 9·3[25] 8·3[26]	—
Collagen	4·7*	—	—	—	—	—
Edestin	2·3	3·22	2·44	2·44[6]	—	—
Gelatin	4·9*	10·1	—	6·2[18]	6·2[20] 4·5[25] 7·3[26]	—
Gliadin	1·2	—	0·79	0·7[19]	—	—
Haemoglobin (horse)	9·8	11·3	10·5	9·43[7]	—	9·8[12]
Insulin	3·1	—	—	1·57[8]	3·2[15]	—
β-Lactoglobulin	13·6	—	13·8	12·1[6]	14·0[15] 13·7[20]	14·0[12]
Myosin	13·7	—	—	11·3[28]	—	—
Ovalbumin	7·4	—	7·45	6·15[6]	8·0[20] 8·1[26]	— —
Salmine	Nil	—	—	—	—	—
Silk fibroin	0·7	—	—	0·25[16]	0·6[17] 0·74[20]	—
Tobacco mosaic virus	1·2	—	—	+[22]	1·64[20]	—
Wool keratin	3·1	—	—	3·12[10]	—	—
Zein	Nil	—	—	Nil	—	—

(*a*) Author, from electrodialysis and N-difference.
(*b*) Albanese[1], from electrodialysis and N-difference.
(*c*) Gale[2], specific decarboxylase applied to hydrolysate.
(*d*) Modifications of picrate method.
(*e*) Microbiological assay.
(*f*) Isotope dilution method.

* Allowance made for hydroxylysine.
Collagen: hydroxylysine-N = 1·1–1·2% protein-N.
Gelatin: hydroxylysine-N = 1·1–1·2% protein-N.

Indices denote references at the end of the paper.

independent enzymic method, is considerably higher, and the histidine of zein is about 50% higher; this figure has not so far been checked by an independent estimation, but it has been obtained on four occasions on two different zein samples. Vickery quotes independent physicochemical data in support of his much lower figure. Lysine values are in general lower by gravimetric methods, but again agreement is fairly satisfactory with the newer procedures. The low gravimetric value for insulin has been discussed by Chibnall; a figure of 3·0 has since been obtained on an insulin catholyte by the picrate method, and it is now believed that the low result obtained by the original procedure was due chiefly to the presence of Na^+ in the lysine fraction, the solubility of lysine picrate being thereby greatly increased. Applying a recent periodate oxidation method to the catholytes, the presence of hydroxylysine has been confirmed (Rees, 1946) in only two of the proteins examined, viz. collagen and gelatin.

It is to be noted that the satisfactory agreement by the newer methods does not extend to the proteins casein and gelatin, where even by the microbiological method, the results are most unsatisfactory. Extreme figures for the lysine content of casein, for instance, range from 8·3 to 10·2. This, however, is not so much a reflexion on the method as an indication of the unsuitability of such proteins as standards for the application of analytical methods. The inconstancy of composition of casein in particular has already been referred to by Chibnall (1946).

In general, however, by the newer methods of analysis, it seems probable that the basic amino-acid figures can now be quoted with a reasonable degree of certainty as to their accuracy, apart from possible losses on hydrolysis. At the same time it has been shown that the standard gravimetric procedures are capable of equal accuracy when applied in favourable circumstances as in the base-

fraction obtained by electrodialysis of a protein hydrolysate.

SUMMARY

1. A scheme of base-analysis is presented consisting of electrodialysis of a protein hydrolysate till free from non-basic-N, thereafter determining arginine and histidine colorimetrically and lysine by N-difference.

2. The results are checked by the application of independent methods to the same base-fraction with satisfactory agreement. The validity of the figures thus depends on the recovery of the bases in electrodialysis and this has been shown to be practically quantitative.

3. The degree of accuracy and the limitations of the various procedures employed have been carefully examined. Both colorimetric methods have been improved and the estimation of arginine by digestion with alkali developed as an accurate micro-method.

4. Analyses are given for reliable samples of 15 proteins of varied origin.

The author wishes to record his particular indebtedness to Prof. A. C. Chibnall for his continued encouragement and criticism which have so materially furthered the progress of this research. Thanks are also due to Dr E. F. Gale, Mr M. W. Rees and Mr E. F. Williams for numerous analyses carried out by them, many of which have not been recorded here. Much of the preliminary work was undertaken in the laboratories of the Animal Diseases Research Association, Moredun Institute, Gilmerton, Midlothian, and the author is grateful to Dr J. R. Greig and Dr J. Stewart of that Institute for their interest in the work.

The tenure of a whole-time grant from the Agricultural Research Council during the course of this work is gratefully acknowledged.

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Studies on the Metabolism of Semen

3. FRUCTOSE AS A NORMAL CONSTITUENT OF SEMINAL PLASMA. SITE OF FORMATION AND FUNCTION OF FRUCTOSE IN SEMEN

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(*Received* 2 *April* 1946)

Previous investigations (Mann, 1945*a*, *b*, *c*) have shown that the glycolytic pathways and enzymes involved in the carbohydrate metabolism of spermatozoa resemble those of other animal tissues and yeast. These studies have established the participation of hexokinase, zymohexase and of certain phosphopherases and dehydrogenases in sperm glycolysis and they disclosed that adenosinetriphosphate (ATP) forms a link between the activity of spermatozoa and glycolysis. In all these experiments washed spermatozoa were used. It remained to be seen what is the glycolytic mechanism which operates in whole semen, i.e. in semen which has not been divided artificially into its two natural components, spermatozoa and seminal plasma.

Since the early researches by Iwanow (1931) and Redenz (1933) it has been known that in one important aspect at least the washed spermatozoa show a strikingly different behaviour from whole semen. Whereas washed spermatozoa can survive anaerobically only in presence of glucose or some other glycolyzable carbohydrate, in whole semen the spermatozoa continue living without additional sugar, at the expense of a reducing and yeast-fermentable carbohydrate already present in the seminal plasma. In the extensive literature dealing with the subject of seminal sugar, this substance has hitherto been described either as glucose or simply as the reducing sugar of semen (Killian, 1933; Bernstein, 1933; Goldblatt, 1935; Shergin, 1937; McKenzie, Miller & Bauguess, 1938; Davis & Cole, 1939; Huggins, Scott, & Heinen, 1942; Moore & Mayer, 1941; MacLeod & Hotchkiss, 1942; Salisbury & Van Demark, 1945), and the only reference to the probable occurrence of fructose in semen is found in a paper by Yamada (1933) who surveyed human tissues and body fluids for the presence of fructose by means of a colour test with 'Cryogenine' (the antipyretic drug 'Cryogénine Lumière').

In view of the inadequate evidence as to the identity of the reducing carbohydrate present in semen, an investigation of its chemical nature was undertaken. The sugar has been purified and shown to be identical with *d*(−)-fructose (preliminary communication, Mann, 1946). In this study it will be demonstrated that fructose accounts for practically the whole of the yeast-fermentable carbohydrate present in the seminal plasma of several species, including bull, ram, rabbit, boar and man. Evidence will also be given that fructose originates in the accessory glands of reproduction and that spermatozoa come in contact with it during their passage through the male generative tract. Finally, it will be shown that fructose in whole semen is the natural substrate which provides life energy to the spermatozoa by its glycolytic breakdown to lactic acid.

EXPERIMENTAL

Methods

The experiments were carried out with ejaculated semen. The semen of animals was collected by Dr A. Walton at the Animal Research Station, Cambridge, by the method of Walton (1945). Several samples of bull semen were kindly supplied by Veterinary Officers in charge of Cattle Insemination Centres in England. The semen was used whole or it was separated by centrifugation into seminal plasma and spermatozoa. The spermatozoa were washed free of the seminal plasma by means of a Ca-free Ringer's solution composed as follows: 100 ml. 0·9% NaCl + 4 ml. 1·15% KCl + 1 ml. 2·11% KH_2PO_4 + 1 ml. 3·82% $MgSO_4.7H_2O$ + 2 ml. 1·3% $NaHCO_3$. In some experiments this solution was further supplemented by 0·5% fructose ('Ringer-fructose') or by 20 ml. 0·25 M-phosphate buffer, pH 7·4

('Ringer-phosphate'), or by both fructose and phosphate ('Ringer-fructose-phosphate').

Total reducing sugar was determined by the method of Hagedorn & Jensen (1923); with this method the reducing value of fructose is only about 2% lower than that of an equivalent amount of glucose (Baranowski, 1935). The value obtained by the reduction of ferricyanide in the Hagedorn-Jensen method will be referred to as the 'total reducing sugar'; it represents the sum of yeast-fermentable sugar and some reducing but non-fermentable substances.

Yeast-fermentable sugar. In order to distinguish between the yeast-fermentable and non-fermentable components of the seminal plasma the following technique was adopted. Fresh baker's yeast (2 g.) was suspended in 20 ml. tap water, left 1 hr. at room temperature and then centrifuged. The yeast was resuspended in 20 ml. of 0·05 M-phosphate buffer (pH 6) or in 20 ml. water. Portions of 2 ml. of the washed yeast suspension were pipetted into two conical centrifuge tubes, *A* and *B*. *A* had no further additions, *B* received 1 ml. seminal plasma. Both tubes were incubated for 2 hr. at 30°. The incubation was terminated by centrifugation, and the centrifuged yeast washed with 2 ml. water. The supernatant solutions and wash-fluids were then transferred quantitatively into two 10 ml. volumetric flasks, and 1 ml. fresh seminal plasma was added to *A*. After diluting the content of each flask with water to the 10 ml. mark, suitable samples (1–5 ml.) were removed and examined for reducing sugar content. The difference in the reducing value between *A* and *B* represents the amount of 'yeast-fermentable sugar'.

The time required for 2 ml. yeast suspension to remove the whole yeast-fermentable sugar from 1 ml. seminal plasma was rather long (up to 2 hr.); much longer than the time needed for yeast to remove the sugar from 1 ml. blood plasma. This is only partly due to the fact that the seminal plasma contains several times more sugar than the blood plasma. The main reason is that the yeast-fermentable sugar present in the seminal plasma is not glucose but fructose. Baker's yeast available locally was found to be much less effective towards fructose than towards glucose.

Fructose. The identification of fructose as the sugar present in the seminal plasma was carried out by the purification of the sugar and by the preparation of the methylphenyl-fructosazone which has been shown by Neuberg (Neuberg, 1902, 1904; Neuberg & Strauss, 1902; Langstein & Neuberg, 1907) to be one of the few chemical derivatives by means of which fructose can be identified and distinguished from glucose and from other sugars. According to Neuberg, pure methylphenyl-fructosazone has m.p. 153°.

For rapid quantitative determinations of fructose in small samples of semen (0·05–0·5 ml.) two colorimetric methods were adapted, those of Roe (1934) and of Stewart, Scarborough & Davidson (1938). Both gave satisfactory results provided that the proportions of the solutions used for deproteinizing, 10% (w/v) $ZnSO_4.7H_2O$ and 0·5 N-NaOH, were carefully adjusted so as to yield mixtures of pH 7·4–7·5. The routine procedure was similar to that used for blood (Somogyi, 1930): The semen was diluted with water to 8 ml., 1 ml. $ZnSO_4$ and 1 ml. NaOH added, the mixture heated for 1 min. in boiling water and filtered; 2 ml. of the clear filtrate were used for fructose estimation. Roe's method could be used also for trichloroacetic acid filtrates.

Glucose. When fructose was established as the principal yeast-fermentable sugar of semen, it became necessary to decide whether fructose occurs in the semen alone or with other yeast-fermentable sugars, particularly glucose, thus requiring a method for the accurate determination of small quantities of glucose in presence of excess of fructose. This was accomplished enzymically by glucose oxidase, an enzyme extracted from moulds, which rapidly oxidises glucose but scarcely attacks fructose. Two sources of the enzyme were used, one a purified preparation of 'Notatin' kindly supplied to Prof. D. Keilin by Prof. Raistrick and Dr Birkinshaw (Keilin & Hartree, 1945); the other a crude but very potent preparation ($Q_{O_2}=1000$) obtained from *Aspergillus niger* in the following manner. The fungus was grown as described by Mann (1944). After 2 days of growth at 30°, 500 ml. filtered culture medium, pH about 4·5–5, were concentrated *in vacuo* to 50 ml., dialyzed overnight, and the enzyme precipitated with 5 vol. of cold acetone. The acetone-dried preparation was stable at 0°. It was dissolved before use in 0·2 M-phosphate buffer, pH 6, so as to give a 0·5% solution. For the estimation of glucose, two incubation flasks were used. Both flasks received 2 ml. seminal plasma + 1 ml. 0·2 M-phosphate buffer, pH 6, and in addition 0·1 ml. glucose oxidase and 1 drop of a catalase preparation were added to one flask. The flasks were then shaken in air at 20° for 2 hr. The samples were then deproteinized and the reducing sugar was determined. The difference in the reducing value between the oxidase-treated and untreated sample gives directly the glucose content in 2 ml. seminal plasma.

Other methods. The methods employed for the estimation of lactic acid and of acid-soluble phosphate were the same as previously described (Mann, 1945*b*, *c*). In a few experiments the 'total carbohydrate' was estimated by the orcinol method of Tillmans & Philippi (1929) and glycogen by the method of Good, Kramer & Somogyi (1933). However, a full interpretation of the data obtained by means of the last two methods will be possible only when the seminal 'glycogen' has been purified and its chemical nature properly investigated.

RESULTS

Purification and identification of fructose in semen

For the purification of fructose bull seminal plasma was chosen because it has an even higher fructose content than the seminal plasma of other mammals. Altogether 120 ml. bull seminal plasma were used, containing 780 mg. fructose. This amount represented a mixture of seven samples, each originating from three to four bulls. The quantitative analysis of the seven samples gave the following results:

Reducing sugar (mg./100 ml.)	785	635	498	447	1090	531	1098
Fructose (colorim.) (mg./100 ml.)	779	592	480	376	1040	435	1062

The seminal plasma was made just acid to methyl red with 0·1 N-HCl and precipitated with 5 vol. of ethanol. The clear filtrate was concentrated by

distillation *in vacuo* at 40° and the thick syrup extracted with several successive portions of boiling ethanol till the residue gave only a very weak Seliwanoff reaction. The combined ethanolic extracts, 250 ml. in all, were concentrated *in vacuo*, and the extraction of the syrup was repeated with boiling ethanol. This extract, on concentration, yielded a syrup which when dissolved in water and filtered through a thin layer of kieselguhr gave 15 ml. of a perfectly clear and almost colourless solution containing 1030 mg. dry matter. Of this material 630 mg., or 65 %, was fructose as assessed by reduction of ferricyanide and by colorimetric determination. This concentrated solution of fructose obtained from seminal plasma was used for the following tests.

Optical activity. A portion was diluted with water to a concentration of 0·848 % reducing sugar, and the solution, examined in the polarimeter in a 2 dm. tube, gave $\alpha = -1{\cdot}56°$, $[\alpha]_D^{20°} = -92{\cdot}2°$. Pure fructose in aqueous solution had $[\alpha]_D^{20°} = -92{\cdot}3°$.

Yeast-fermentation test. A 1 ml. sample (42 mg. seminal fructose) suitably diluted with phosphate buffer, pH 6, was divided into two portions of which one was incubated for 2 hr. with yeast, the other serving as control. In both samples the reducing sugar and fructose were determined. The yeast-incubated sample no longer contained any fructose or reducing sugar.

Absence of glucose. 1 ml. suitably diluted was examined for the presence of glucose by means of glucose oxidase but there was no change in either reducing value or fructose content as the result of this treatment.

Phenylosazone. 1 ml. was used for the preparation of phenylosazone. Two recrystallizations from ethanol gave 19 mg. of osazone; m.p. 205°.

Methylphenyl-fructosazone. The remaining solution of seminal fructose was concentrated *in vacuo* over P_2O_5 to 3·3 ml. Insoluble material was centrifuged off, and 3 ml. of the clear solution (450 mg. reducing sugar) was treated in a small tube with 1 g. α-methylphenylhydrazine and then ethanol added drop by drop till a clear solution was obtained. After addition of 1 ml. 50 % acetic acid the tube was immersed in boiling water for 5 min. and then cooled in ice. Within 30 min. there appeared a mass of yellow needle-shaped crystals. In addition, a brown oily material was deposited in the bottom of the tube. After 2 hr. the crystalline material was separated from the oil, and washed on a sintered glass filter with ice-cold water. It was recrystallized from 15 % (v/v) ethanol giving 200 mg. of the recrystallized osazone; m.p. 151°. Methylphenylosazone prepared from pure fructose had exactly the same m.p. (151°). An attempt to obtain in this manner methylphenylosazone from glucose was negative.

A further batch of methylphenylosazone was obtained from the oily residue. This was washed several times with cold water (by decanting), dried over H_2SO_4 and then dissolved in absolute ethanol. The solution (3 ml.) was filtered into a test-tube and immersed in a freezing mixture of acetone and solid carbon dioxide. The crystallization began almost at once and after 14 hr. at 0° the crystals were separated. A little more crystalline osazone was obtained from the filtrate by gradual addition of water. However, the product obtained in this manner had m.p. 148°, even after one recrystallization. The total yield of methylphenyl-fructosazone from the seminal sugar was 56 %. The yield obtained by Neuberg & Strauss (1902) from ascites fluid was 66 %; they reported even higher values in some of their other preparations.

Search for other sugars in seminal plasma

Phosphofructose. Bull seminal plasma contains more fructose than the plasma of other animals. It is also distinguished by a remarkably high though variable content of organic acid-hydrolyzable phosphorus compounds (130–200 mg. P/100 ml.). The possibility was envisaged that some of this organic phosphorus may occur in the form of phosphofructose. However, the presence of more than a negligible quantity of fructose in the phosphorylated form may be discounted in view of the following facts. When the seminal plasma is treated with yeast (2 hr., 30°) only 2–25 mg./100 ml. fructose remain out of several hundred mg./100 ml. fructose originally present, yet the disappearance of fructose is not accompanied by any appreciable diminution in the above-mentioned organic P-fraction. Moreover, if the seminal plasma is precipitated with Ba acetate in presence of ethanol, the precipitate contains very little fructose, practically all of which remains in the supernatant solution. Under these conditions monophospho- and diphospho-fructose would both be precipitated as Ba-salts.

The possibility was also considered that apart from fructose the seminal plasma may contain some ketotrioses, either phosphorylated or phosphate-free, which give the same colour reactions as fructose and its derivatives. However, this appeared unlikely in view of the very low content of bisulphite-binding substances. Trichloroacetic acid extracts from bull and ram seminal plasma treated with $NaHSO_3$ according to Clift & Cook (1932) used up less than 0·2 ml. 0·01 N-I_2/ml. semen.

Glucose. Yet another question to be considered was that the seminal plasma might contain some glucose in addition to the large amount of fructose. Table 1 shows that this can be ruled out. When the seminal plasma is shaken aerobically with glucose oxidase and the reducing value of such a sample is compared with that of fresh seminal plasma, the

difference found is very small indeed. Glucose added to seminal plasma disappears after treatment with glucose oxidase.

Table 1. *The effect of glucose oxidase on total reducing sugar and fructose content of seminal plasma*

(Total reducing sugar estimated by reduction of ferricyanide; fructose determined colorimetrically. Results expressed in terms of mg. fructose/100 ml. seminal plasma.)

	Before incubation with glucose oxidase		After incubation with glucose oxidase	
	Reducing sugar	Fructose	Reducing sugar	Fructose
Bull:				
Sem. plasma	559	513	546	505
Sem. plasma +0·8% glucose	1359	539	546	535
Ram:				
Sem. plasma	682	565	682	565
Sem. plasma	231	191	220	190
Rabbit:				
Sem. plasma	618	515	610	515
Blood serum	142	—	15	—
Blood serum +1% glucose	1142	—	16	—
Man:				
Sem. plasma	265	100	257	100
Boar:				
Sem. plasma	31	9	30	9

Concentration of fructose in seminal plasma of various animals

The quantitative assay of fructose was carried out in samples of semen from various animals, ranging from 0·05 ml. in bull to 1 ml. in boar. In view of the identification of fructose by the isolation of methylphenylosazone and by other means as described in the preceding parts, it was safe to regard the values obtainable by colorimetric methods as representing fructose. Whenever practicable, the determination of reducing sugar was carried out simultaneously with the colorimetric estimation of fructose; in all such experiments the same standard solution of pure fructose was used for both methods.

Comparative figures for total reducing sugar, yeast-fermentable sugar and fructose in the seminal plasma of several species in Table 2 reveal the strikingly high level of fructose, which in bull, ram, rabbit and man exceeds several times that of glucose in blood. In boar, on the other hand, only 9 mg./100 ml. fructose was found, or about 1/100 of the fructose concentration in bull. However, it must be remembered that the volume of a single ejaculate of boar (250–500 ml.) is almost 100 times larger than that of bull so that in both animals the quantity of fructose contained in a single ejaculate is about the same: 20–50 mg.

Table 2. *Total reducing sugar, yeast-fermentable sugar and fructose content of seminal plasma*

(Results expressed as mg. fructose/100 ml. seminal plasma.)

	Total reducing sugar	Fructose	Yeast-fermentable sugar	Non-fermentable residue
Bull	785	779	779	16
	1090	1040	1055	35
Ram	458	340	350	108
	315	268	269	46
	472	285	394	78
	525	466	466	59
Rabbit	438	318	300	138
	618	515	521	97
Man	260	100	100	160
	262	120	128	134
	362	204	208	154
Boar	31	9	10	21

There is on the whole a close agreement between the values for fructose and for yeast-fermentable sugar. This furnishes an additional proof that glucose, if at all present, occurs in the seminal plasma only as a slight admixture with fructose. However, occasionally a discrepancy has been observed between the content of yeast-fermentable sugar and that of fructose, indicating the presence of a small amount of yet another yeast-fermentable component. On the other hand, there is in almost every instance a distinct difference between the total reducing sugar and the yeast-fermentable sugar; this points to the existence in the seminal plasma of some reducing but non-fermentable substances. The chemical nature of these substances remains to be investigated.

Another characteristic feature of the seminal sugar is the variability of the fructose content in the semen of the same species. Considerable variations were observed in the semen from the same animal collected on different occasions, in spite of the fact that all estimations of fructose were carried out as soon after ejaculation as possible. These findings agree with statements by earlier investigators who recorded similar variations in reducing sugar in semen (Goldblatt, 1935; Shergin, 1937; McKenzie *et al.* 1938; Davis & Cole, 1939; Ross, Miller & Kurzrok, 1941; Moore & Mayer, 1941). The variations in the content of seminal fructose are difficult to explain. If they were solely due to fructolysis taking place in semen already before ejaculation, i.e. inside the male body, one would expect that samples of semen comparatively poor in fructose would have at the same time a rather high content of lactic acid. This, however, is not so (Table 3). It seems, therefore, that the level of fructose in the semen is not controlled by that mechanism which so strictly regulates the level of glucose, for instance, in blood and some other body fluids.

Table 3. *Fructose and lactic acid content of whole semen (ram)*

(Semen deproteinized within 10 sec. after ejaculation.)

Ram no.	Fructose (mg./100 ml.)	Lactic acid (mg./100 ml.)
1	188	43
	274	25
2	280	43
	428	56
3	238	42
4	300	37
5	176	22
6	340	55
	500	62

Fructose in accessory organs of reproduction

As shown above, freshly ejaculated semen of ram or bull contains several hundred mg./100 ml. fructose. If this high content of fructose is connected with the presence of spermatozoa as such, one would expect in the testes and in the epididymis an even higher concentration of fructose. Yet both these organs were found to contain only 1–4 mg./100 ml. fructose. Moreover, the epididymal semen, i.e. the thick suspension of spermatozoa which can be obtained directly from the ram or bovine epididymis, gives a barely perceptible Seliwanoff reaction. This shows that the site of fructose formation in the male body is situated along the generative passage between the epididymis and the urethral outlet.

Huggins & Johnson (1933), to whom we owe an extensive study of the secretions produced by the prostate gland and the seminal vesicles in man, have noted that the high concentration of reducing sugar in the semen is due principally to the seminal vesicles. Similar observations were made on the bull (Bernstein, 1937), boar (McKenzie *et al.* 1938) and ram (Moore & Mayer, 1941). In view of these observations it seemed desirable to determine the fructose content in the various accessory glands of reproduction. Table 4 shows that in the bull and ram the seminal vesicles contain a high concentration of fructose. On the other hand, in rabbit, which does not possess seminal vesicles, the prostate (Gl. vesicularis) is rich in fructose. The form in which fructose occurs in the accessory glands is under investigation.

Table 4. *Distribution of fructose in organs of reproduction*

	Fructose (mg./100 ml.)				
	Bull	Ram	Rat	Rabbit	Cat
Testes	3	1	1	1	3
Epididymis	3	3	1	1	4
Prostate gland	5	8	10	230	35
Seminal vesicles	480	160	10	—	—
Fluid from seminal vesicles	840	570	—	—	—
Ampullae	—	—	—	12	—
Uterus masculinus	—	—	—	0	—
Cowper's gland	—	—	—	Trace	—

Function of fructose in semen

The main function of fructose is to supply the life energy to the spermatozoa in the form of an easily glycolyzable material. The incubation of a freshly ejaculated whole semen is followed by a progressive fall in fructose, and lactic acid accumulates as the result of fructolysis (Table 5). The rate of fructolysis is greater in N_2 than in air, and the ratio between lactic acid produced and fructose used up is appreciably higher in N_2 than in air (Table 5, Exp. 1). Aerobically, fructolysis is not the only source of energy for the spermatozoa which, even if deprived of fructose, can survive in O_2 owing to utilization of other substances mainly of undetermined nature. Anaerobically, however, the spermatozoa very largely depend on fructose and the cessation of fructolysis invariably terminates their activity. In order to deplete ram semen of most of its fructose it is usually enough to incubate the semen in N_2 for a few hours at 30–37°. If the anaerobic fructolysis is allowed to proceed almost to depletion of fructose and if at this point fresh 'Ringer-fructose-phosphate' is added, this enables the spermatozoa to retain their anaerobic activity and to continue to produce lactic acid with unabated vigour (Table 5, Exp. 2). Reinforcement of the fructose content by extra fructose added at the start of the incubation period has a similar effect (Table 5, Exp. 3). It must be stressed, however, that the survival under anaerobic conditions in semen can only be achieved if the pH of semen is not allowed to fall too low; for this reason sugar was added to semen in the form of 'Ringer-fructose-phosphate' solution which has a high buffering capacity.

Since there is such a close connexion between the disappearance of fructose and formation of lactic acid one might expect perhaps that the addition of excess carbohydrate to whole semen would not only prolong the duration of fructolysis but would at the same time enhance the rate of lactic acid formation. However, that is not so, at least not in semen which already has a high concentration of fructose. It can be seen from Exp. 3, Table 5, that during the first hour of anaerobic incubation, the yield of lactic acid formed is not markedly affected by the presence of additional sugar. Only later, when the semen has depleted its own sugar reserve, the beneficial effect of extraneous sugar becomes more apparent. In Table 6 fructose disappearance in whole semen is compared with lactic acid formation both in absence and in presence of various additional sugars. It can be seen that in presence of

Table 5. *Fructolysis in whole semen*

Exp. 1. Ram semen incubated at 30°; anaerobically in Thunberg tubes filled with N_2; aerobically by shaking in Barcroft manometers filled with air.

	Incubation (min.)	Fructose (mg./100 ml.)	Lactic acid (mg./100 ml.)	Fructose loss accounted for as lactic acid (%)
	0	490	112	—
Anaerobically	150	312	290	100
	300	160	396	86
	450	41	500	86
Aerobically	150	336	220	70
	300	215	292	65
	450	94	336	42

Exp. 2. Ram semen; anaerobic incubation at 37°.

	Additions to semen	Incubation (min.)	Fructose (mg./100 ml.)		Lactic acid (mg./100 ml.)	
			Content	Decrease	Content	Increase
Fresh semen	—	0	465	—	118	—
	—	120	35	430	394	276
Same semen after 120 min. incubation	1 vol. Ringer-phosphate	0	35	—	394	—
		90	9	26	407	17
	1 vol. Ringer-fructose-phosphate	0	535	—	394	—
		90	287	248	593	199

Exp. 3. 1 ml. samples of semen diluted with 1 ml. Ringer-phosphate (R. phosph.), Ringer-fructose-phosphate (R. fruct. phosph.) and Ringer-phosphate containing 0·5% glucose instead of fructose (R. gluc. phosph.), respectively. Otherwise conditions as in Exp. 2.

Additions to semen	Incubation (min.)	Fructose (mg./100 ml.)		Lactic acid (mg./100 ml.)	
		Content	Decrease	Content	Increase
—	0	393	—	127	—
R. phosph.	60	57	336	284	157
	120	42	351	291	164
	180	9	384	289	162
R. fruct. phosph.	60	582	311	161	161
	120	450	443	240	240
	180	389	504	297	297
R. gluc. phosph.	60	318	75	164	164
	120	265	128	236	236
	180	187	206	315	315

Table 6. *Effect of additional carbohydrate on the rate of glycolysis and on the fructose content of whole semen*

(1% carbohydrate added to whole semen and mixture incubated at 30°.)

Carbohydrate added to whole semen	Incubation (hr.)	Fructose (mg./100 ml.)	Lactic acid (mg./100 ml.)	Decrease in fructose (%)	Ratio: Lactic acid formed / Fructose broken down
None	0	331	201	0	—
None	3	41	397	88	0·67
Glucose	3	274	390	18	3·30
Fructose	3	—	432	—	—
Mannose	3	277	394	17	3·60
Glycogen	3	43	405	87	0·72
Galactose	3	59	369	82	0·61
Pyruvate	3	28	560	92	1·18
Arabinose	3	31	351	91	0·50
Maltose	3	128	401	62	0·99
Lactose	3	131	393	61	0·96
Sucrose	3	—	390	—	—
Raffinose	3	—	371	—	—

added glucose or mannose the rate of fructose disappearance from semen is considerably diminished, but the rate of lactic acid formation remains unimpaired. In other words, the addition of glucose or mannose to whole semen has a suppressing influence on the lactic acid formation from fructose, but this decrease is made good by lactic acid produced from glucose or mannose respectively. The 'preserving effect' on the seminal fructose is evident only with glycolyzable sugars such as glucose and mannose. Glycogen and other non-glycolyzable sugars have no effect.

The rate of fructolysis in semen is highly dependent on temperature (Table 7). At 5–10°, which is the usual temperature for storing semen, an appreciable proportion of seminal fructose still remains after 2 days' incubation. At 30–37°, on the other hand, the disappearance of fructose is only a matter of a few hours.

Table 7. *Effect of temperature on the rate of fructolysis in whole semen*

(Ram semen stored in narrow test-tubes at constant temperature.)

Exp. 1

Incubation Temp.	Incubation Hr.	Fructose (mg./100 ml.)	Lactic acid (mg./100 ml.)	Decrease in fructose (%)	Fructose loss accounted for by lactic acid (%)
—	—	414	204	0	—
5°	48	335	242	18	48
10°	48	113	278	77	25
24°	4	125	364	70	55
30°	3	83	421	80	69
37°	2	50	423	87	60

Exp. 2

Incubation Temp.	Incubation Hr.	Fructose (mg./100 ml.)	Decrease in fructose (%)
—	0	265	0
5°	1	259	4
10°	1	254	8
15°	1	222	17
24°	1	40	65
28°	1	11	95
30°	1	10	96

Apart from fructose, the semen contains some reducing substances which are not fermented by yeast as well as a certain amount of carbohydrate material which resembles glycogen. So far, however, no evidence could be produced that any of these compounds can be utilized by the spermatozoa as an additional source of glycolysis (Table 8).

The seminal 'glycogen', i.e. the alkali-resistant polysaccharide which behaves on ethanol-precipitation and hydrolysis like the glycogen of other animal tissues, seldom exceeds 0·1 % in ram semen; of seven samples of ram semen, in five the glycogen content ranged from 0·019 to 0·078 %, the remaining two had 0·118 and 0·190 % respectively. Even on prolonged incubation at 37°, under conditions where fructose is completely exhausted, the level of glycogen in semen underwent little change. The chemical properties and function of seminal glycogen remain to be investigated.

With regard to the enzymic mechanism in semen which regulates the fructose metabolism, this is similar in many ways to the mechanism of glucose metabolism previously described in studies on washed spermatozoa (Mann, 1945*c*). There it was shown that glucose is first phosphorylated by adenosinetriphosphate and that the monophosphohexose thus formed is further metabolized through diphosphofructose, phosphotriose, phosphoglyceric acid and pyruvic acid to lactic acid. Table 9 shows that glucose, fructose and mannose are metabolized by washed spermatozoa at a rate which is of an equally high order for all three sugars; galactose and glycogen are glycolyzed only very slowly. Also, the hexokinase reaction can be demonstrated for glucose, fructose and mannose alike when these sugars are incubated with washed spermatozoa in presence of fluoride and adenosine triphosphate (ATP). Table 10 shows that almost half of the readily hydrolyzable phosphorus of ATP is esterified within ½ hr. at 30°, and to the same extent with glucose, fructose and mannose. Among the various other sugars examined, only galactose seems to undergo esterification with ATP. However, a small degree of esterification with ATP can be observed even without additional sugar (Exp. 2, Table 10). This is probably due to small amounts of carbohydrate present in the spermatozoa despite the washing.

The hexokinase reaction also takes place in whole semen (Table 11), the chief reactants being fructose contained in the seminal plasma and preformed ATP in the spermatozoa. As a result of this reaction phosphohexose accumulates and can be precipitated as Ba-salt at pH 8 in the presence of 80 % ethanol. Emphasis must be laid upon the peculiar dependence which was formerly shown to exist between the level of ATP and the glycolysis in washed spermatozoa. This dependence is an equally outstanding feature in whole semen; here, too, the maintenance of the ATP level is intimately linked, at any rate anaerobically, with the unhampered progress of glycolysis (Table 12).

DISCUSSION

Generally, the evidence for the occurrence of free fructose in the animal body has been rather scanty. The presence of fructose in the embryo is one of the few instances where the nature of this sugar was adequately proved. A laevorotatory constituent in foetal fluids was first noticed by Claude Bernard (1855) but its identity with fructose was recognized

Table 8. *Changes in various carbohydrate fractions brought about by anaerobic glycolysis in whole semen*

Exp. 1. 10 ml. ram semen divided into 2 equal parts, A and B. A centrifuged immediately; B centrifuged after 3 hr. incubation at 37° in N_2. Centrifuged seminal plasma subdivided into 2 equal parts, one examined without further treatment, and the other after 2 hr. treatment with washed yeast. Results expressed in mg./100 ml. seminal plasma.

	A Seminal plasma from fresh semen			B Seminal plasma from semen allowed to glycolyze for 3 hr., 37°, in N_2		
	Yeast-fermentable	Non-fermentable	Together	Yeast-fermentable	Non-fermentable	Together
Total carbohydrate in non-deproteinized seminal plasma	508	370	878	51	368	419
Total carbohydrate in protein-free filtrates	491	170	661	49	167	216
Total reducing sugar in protein-free filtrates	457	78	535	19	97	116
Fructose	447	19	466	21	11	32

Exp. 2. 20 ml. ram semen divided into 2 equal parts, A and B. A used fresh; B after 3 hr. incubation at 37°, in N_2. Glycogen estimated in 5 ml. samples of whole semen. Free fructose and 'Embden ester' estimated in filtrates after treatment of whole semen with trichloroacetic acid. The 'Embden ester' was separated as follows. Ba acetate added at pH 8, precipitate discarded, supernatant solution treated with 5 vol. ethanol, left overnight, and centrifuged. The precipitate dissolved in dilute HCl, Ba removed with Na_2SO_4, the Ba-free extracts analyzed. Results in mg./100 ml. whole semen.

	A Fresh semen	B Semen allowed to glycolyze for 3 hr. at 37°
Free fructose	330	23
Glycogen:		
Reducing sugar	118	101
'Embden ester':		
Reducing sugar	21	36
Fructose	3	6
P_0	0	0
P_{30}	1	2
$P_{tot.}$	7	12

(P_0 = phosphorus determined as phosphate which reacts directly with ammonium molybdate; P_7 and P_{30} = phosphorus which appears as orthophosphate after 7 and 30 min. hydrolysis with N-HCl; $P_{tot.}$ = phosphorus as total phosphate after incineration of trichloroacetic acid extract.)

only some time later; fructose was definitely shown to be a normal constituent of both the allantoic and amniotic fluid and in urine of newly-born animals (Majewski, 1858; Grüber & Grünbaum, 1904; Paton, Watson & Kerr, 1907; Langstein & Neuberg, 1907); it was also stated to be present in foetal blood (Orr, 1924). Fructose has also been demonstrated in certain pathological dysfunctions like fructosuria and in transudates (Rosin & Laband, 1902; Neuberg & Strauss, 1902; Moraczewski, 1907; Adler, 1911; Barrenscheen, 1922). In most, if not in all of these instances, the concentration of fructose was sufficiently high to make practicable a proper identification of the sugar by such means as purification, optical activity measurements and the preparation of specific derivatives. Occasionally, however, claims were made of the alleged presence of fructose in animal tissues and body fluids where the chemical criteria applied were less satisfactory. Not infrequently statements were based merely on positive reactions with certain colour-producing substances such as resorcinol (Seliwanoff, 1887), diphenylamine (Ihl, 1885) and cryogenine (Yamada, 1933). Unfortunately, these colour reactions, unless supplemented by more direct and specific methods, seldom disclose the true chemical nature of the sugar studied, since they give positive results not only with fructose but also with a variety of other substances such as the Neuberg ester (6-phosphofructose), Harden-Young ester (2:6-diphosphofructose), sucrose, methylglyoxal, dihydroxyacetone, and numerous ketoses and ketose derivatives.

Among the body fluids, semen is distinguished by a very high content of reducing sugar which the present study proves to be *d*(−)-fructose. The purified seminal sugar shows the same reducing power and the same specific optical activity as pure fructose, and it yields the crystalline methylphenylfructosazone typical of fructose. It also gives the phenylosazone and it is yeast-fermentable but,

Table 9. *Glycolysis in washed spermatozoa*

(Spermatozoa washed free from seminal plasma and made up to the original vol. of whole semen with Ringer-phosphate.)

Exp. 1. Bull spermatozoa, incubated 30 min. at 30° with 0·02 M-sugar.

	Lactic acid (mg./100 ml.)
No substrate	1·5
Anaerobically:	
Glucose	30·5
Fructose	42·5
Mannose	30·5
Aerobically:	
Glucose	24·5
Fructose	24·5

Exp. 2. Ram spermatozoa, incubated anaerobically for 2 hr. at 37° with 0·1 M-sugar.

	Lactic acid (mg./100 ml.)
No substrate	7·0
Glucose	447·0
Fructose	417·0
Mannose	405·0
Galactose	103·0
Glycogen	32·0

unlike glucose, it is not acted upon by glucose oxidase. In addition, it reacts in a characteristic manner with the Seliwanoff reagent and with other ketose-reagents.

At the site of their origin, in the testes and in the epididymis, the spermatozoa, still immotile, have hardly any fructose at their disposal. However, when they traverse the male generative passages from the testes onwards they receive the complementary secretions of several accessory organs of reproduction—seminal ducts, seminal vesicles, prostate gland, Cowper's gland and other urethral glands. These secretions, which together constitute the seminal plasma, form the natural environment and nutrient medium for the reproductive cells. Fructose is one of the numerous constitutents of the seminal plasma. Its concentration varies greatly but in some species it may amount to as much as 1 g./100 ml. plasma. The seminal vesicles are the main contributors of fructose and the final concentration of fructose in the whole semen as ejaculated largely depends on the contribution made by these organs. In some animals, which do not possess seminal vesicles, e.g. rabbit, part of the prostate provides the source of fructose. In the semen itself fructose occurs in a free, non-esterified form. This, however, does not exclude the possibility that fructose may be the end-product of enzymic reactions involving more complex compounds.

Once in contact with spermatozoa, fructose diffuses readily into the cells and enters the characteristic chain of reactions initiated by the hexokinase-catalyzed reaction with adenosinetriphosphate, leading finally to lactic acid. However, even under purely anaerobic conditions the production of lactic acid does not account for the total fructose metabolized. In this respect semen behaves like other animal tissues, except that whereas the majority of tissues have glucose or glycogen at their disposal, semen uses fructose which is the naturally available sugar. It should be pointed out, however, that although normally spermatozoa utilize fructose they are well able to glycolyze other sugars as well. If glucose or mannose, for instance, is added to the semen, this offers the spermatozoa a choice of several glycolyzable substrates and they actually make use of all of them so that whereas in untreated semen the entire lactic acid would have been the outcome of fructolysis, in presence of additional sugar only a certain proportion of lactic acid is derived from the seminal fructose and the rest is the result of glycolysis of the extraneous sugar. This

Table 10. *Phosphorylation of carbohydrate by adenosinetriphosphate in washed sperm*

Exp. 1. 10^9 ram spermatozoa + 0·422 mg. pyro-P of ATP + 0·05 M-sugar + 0·04 N-NaF; anaerobic incubation 30 min. at 30°, in 95% N_2 + 5% CO_2.

	Incubation (min.)	P_0 (mg.)	P_7 (mg.)	P_{30} (mg.)	Pyro-P esterified with sugar (mg.)	(%)
No sugar	0	0·198	0·620	0·626	0	0
Glucose	30	0·251	0·428	0·521	0·182	43
Fructose	30	0·251	0·418	0·500	0·202	48
Mannose	30	0·261	0·475	0·525	0·145	35

Exp. 2. 10^9 ram spermatozoa + 0·283 mg. labile P of ATP + 0·03 M-sugar + 0·05 N-NaF; incubation in test-tubes open to air, 30 min. at 30°.

	Incubation (min.)	P_0 (mg.)	P_7 (mg.)	Pyro-P esterified with sugar (mg.)	(%)
No sugar	0	0·222	0·505	0	0
No sugar	30	0·272	0·495	0·011	4
Glucose	30	0·243	0·381	0·124	44
Fructose	30	0·222	0·376	0·129	45

Exp. 3. 10^9 ram spermatozoa + 0·251 mg. pyro-P of ATP + 0·03 M-sugar + 0·04 N-NaF; incubation in test-tubes open to air, 30 min. at 30°.

	Incubation (min.)	P_0 (mg.)	P_7 (mg.)	Pyro-P esterified with sugar (mg.)	(%)
No sugar	0	0·244	0·495	0	0
No sugar	30	0·282	0·447	0·048	19
Glucose	30	0·232	0·383	0·112	44
Fructose	30	0·225	0·369	0·156	62
Mannose	30	0·251	0·388	0·107	42
Galactose	30	0·252	0·398	0·097	39
Arabinose	30	0·294	0·466	0·029	11
Sucrose	30	0·293	0·473	0·022	9
Maltose	30	0·294	0·478	0·017	6
Raffinose	30	0·294	0·448	0·047	19
Lactose	30	0·318	0·478	0·017	6

Table 11. *Phosphorylations in whole semen*

Exp. 1. Ram semen diluted with equal volume of 'Ringer-fructose-phosphate' and incubated at 30°, then centrifuged and the sperm extracted with trichloroacetic acid. Results are expressed in mg. P/100 ml. non-diluted semen; NaF, 0·02N; iodoacetate (IAA), 0·001N.

Additions to semen	Incubation min.	P_0	P_7	P_{30}	$P_{alk.}$	Pyro-P esterified with sugar	P_{30}–P_7	$P_{alk.}$–P_0
0	20	8·0	15·7	16·5	8·0	0	0·8	0·0
NaF	20	8·0	11·6	13·6	8·0	4·1	2·0	0·0
IAA	20	11·2	13·2	15·9	12·8	2·5	2·7	1·6

Exp. 2. Incubated 30 min. at 37°: (A) 5 ml. ram semen without any additions, (B) 5 ml. ram semen containing 0·02N-NaF. Incubation terminated by centrifugation. Centrifuged sperm extracted with trichloroacetic acid. Extracts precipitated with Ba acetate at pH 8 in presence of 10% ethanol, precipitates discarded. Supernatant solutions treated with 5 vol. of ethanol, the precipitate analyzed for P-fractions and fructose (from phosphofructose). Results in mg./100 ml. semen.

	P_0	P_7	P_{30}	$P_{tot.}$	$P_{tot.}$–P_{30}	Fructose
A	0	0	0·10	3·05	2·95	0·2
B	0	0·50	0·70	6·15	5·45	3·4

($P_{alk.}$ = alkali labile P = phosphorus which appears as orthophosphate after exposure to 0·1N-NaOH for 20 min. at 20°; P_0, P_7, P_{30} and $P_{tot.}$ = symbols explained in footnote to Table 8.)

Table 12. *Dependence of the ATP-level on the fructolysis in whole semen*

Exp. 1. 150 min. anaerobic incubation at 37°.

Inhibitor added to ram semen	Anaerobic incubation at 37° (min.)	Fructose (mg./100 ml.)	Lactic acid (mg./100 ml.)	ATP-amino-N (mg./100 ml.)
—	—	432	158	1·44
—	150	38	346	0·74
0·015N-NaF	150	168	158	0·28
0·001N-iodo-acetate	150	248	128	0·35

Exp. 2. 10 ml. ram semen allowed to glycolyze 2 hr. at 37°, i.e. till the fructose content was reduced to 2 mg. Then divided into two equal parts of which one (A) was diluted with 1 vol. Ringer-phosphate, and the other (B) with 1 vol. Ringer-phosphate containing 27·8 mg. fructose. Deproteinized with trichloroacetic acid after 2 hr. incubation at 37°. Results are expressed in mg./100 ml. semen.

	Fructose		Lactic acid	ATP-amino-N
	Before incubation	After incubation	After incubation	After incubation
A	2	0	10·2	0·31
B	29·8	16·7	22·6	0·92

explains why it has been possible in the past to employ glucose successfully as the nutrient component of artificial media for storing mammalian semen. The ability of spermatozoa to make equal use of fructose, glucose and mannose has its foundation in the fact that all three sugars enter the cycle of glycolysis by way of the same hexokinase-reaction with ATP, followed by the formation of monophosphohexose. From this point onwards the breakdown leads through diphosphofructose, phosphotriose, phosphoglyceric acid, pyruvic acid to lactic acid, and in this respect the process in whole semen is very much like that in washed spermatozoa to which sugar was added artificially (Mann, 1945*a*, *b*, *c*).

The progress of fructolysis in semen depends on several factors, such as the actual concentration of fructose in the seminal plasma, the density of spermatozoa, pH and temperature. In the field of artificial insemination it has been a practice of long standing to store the semen of farm animals at a comparatively low temperature of 5–10°, since at this temperature the spermatozoa were known to remain alive much longer than at body temperature. It is now probable that this is due to the slower rate of fructose utilization at lower temperature.

At present it is difficult to form an opinion as to why the animal body should choose fructose instead of glucose or glycogen as the natural sugar of semen. One may recall at this point another body fluid, milk, where the occurrence of lactose creates a similar problem (Kay, 1945). There may be, of course, several causes for the presence of fructose in semen but at least one already established fact must be taken into consideration. This relates to the rather specific behaviour of spermatozoa towards fructose as compared with that of most other animal tissues, few of which, according to Dickens & Greville (1932, 1933) who made a thorough comparative study of glucose and fructose metabolism in numerous animal organs, are capable of metabolizing fructose anaerobically to lactic acid. This should enable the spermatozoa to draw freely on the fructose reservoir as provided by the organs of reproduction without any serious competition from other animal tissues.

SUMMARY

1. Contrary to the views generally held, it was found that the reducing carbohydrate of the seminal plasma is not glucose but *d*(−)-fructose.

2. Fructose has been purified from seminal plasma and identified by its reducing value, optical activity, preparation of methylphenyl-fructosazone, yeast-fermentation test and by other means.

3. In the seminal plasma of several species, including bull, ram, rabbit, boar and man, fructose accounts for practically the whole of the yeast-fermentable reducing sugar. Little glucose, if any, is present in the seminal plasma; this was shown by applying a method which makes use of mould glucose oxidase which oxidizes glucose quantitatively but leaves fructose untouched.

4. The level of fructose in seminal plasma varies from one species to another and even within the same species there are individual differences. The highest values were observed in bull where the concentration sometimes exceeds 1 g. fructose/100 ml. seminal plasma.

5. Sperm obtained directly from the epididymis contains hardly any fructose. During the passage through the male generative tract the semen acquires fructose from the accessory glands of reproduction, of which the seminal vesicles are the chief contributors of fructose. In some animals which have no seminal vesicles proper, fructose is generated in the prostate.

6. The main function of fructose in semen is to supply the spermatozoa with readily glycolyzable material. On storage, the content of seminal fructose falls progressively and lactic acid accumulates.

7. Normally spermatozoa utilize fructose, as this is the chief sugar available in seminal plasma, but their enzymic equipment enables them to metabolize equally efficiently glucose and mannose. The glycolysis of these three sugars in sperm is initiated by a hexokinase interaction with adenosinetriphosphate. The monophosphohexose thus formed is further metabolized through diphosphohexose, phosphotriose, phosphoglyceric acid and pyruvic acid to lactic acid.

This work has been carried out on behalf of the Agricultural Research Council. I wish to thank Prof. D. Keilin, F.R.S., and Dr A. Walton for their valuable help and kind interest in this work. For the collection of the material I am greatly indebted to Dr A. Walton and to the following veterinary officers in charge of Cattle Insemination Centres in England: Mr R. Clarke, Mr E. D. Conn, Mr L. E. A. Rowson, Mr G. Smith and Mr D. L. Stewart.

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Palm-oil Carotenoids

5. EXAMINATION OF 'SESE' PALM OILS

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(*Received* 8 *April* 1946)

The palm oil from plantations of the Sese district of the Gold Coast is one of the most highly coloured African palm oils. It was therefore of interest to examine the carotenoids present in commercial samples of 'Sese' oil and in oils especially extracted from selected fruits.

Examination of samples of 'Sese' oil obtained from bunches of fruit of the *Edibe*, *Abepa*, and *Lisombe* varieties on the lines of earlier investigations (Hunter & Scott, 1941, 1944) furnished crystalline specimens of α and β-carotene and spectroscopic evidence of the presence of γ-carotene, *neo*-γ-carotene, lycopene, and *neo*-lycopene in all three cases. Small crystalline specimens of lycopene and γ-carotene were isolated from the *Lisombe* oil. Chromatographic separation of the ether-soluble unsaponifiable matter from *Abepa* oil furnished a small quantity of lutein (cf. Hunter, Scott & Edisbury, 1942).

A commercial sample of ordinary Sese oil yielded crystalline specimens of α, β, and γ-carotenes and a reasonably pure specimen of lycopene. Spectroscopic evidence was obtained of the presence of *neo*-γ-carotene, *neo*-lycopene, and *neo*-lutein. It may be mentioned, however, that one commercial sample of 'Sese' palm oil which was examined some years ago (Hunter & Jones, 1940) furnished a substantial amount of a carotenoid which separated on the chromatogram at the position corresponding to cryptoxanthin and had the spectroscopic characteristics of this monohydroxy-β-carotene. The presence of this carotenoid has not been observed in any other sample of 'Sese' palm oil subsequently examined. With the exception of one sample therefore, 'Sese' palm oil shows a close similarity to the other palm oils with regard to the carotenoids present.

EXPERIMENTAL

Edibe oil. The sample showed an extinction coefficient of 3·13 at 460 mμ. in benzene. A carotene estimation made on 100 g. of oil by the method described by Hunter & Scott (1944) gave 0·0977% of carotene (α+β). The dextrorotation of the mixture of isomers in benzene was 230°, corresponding to 33% of the α-isomer.

The oil (350 g.) was saponified with KOH (105 g.) in water (175 ml.), ethanol (550 ml.) and light petroleum (350 ml.). The mixture was heated until a clear solution was obtained, kept, heated under reflux for an hour, cooled, diluted with ethanol (1225 ml.) and water (2650 ml.) and extracted with light petroleum (1500 ml.). The extract was washed, dried with anhydrous $MgSO_4$, and evaporated with the usual precautions.

The petrol-soluble unsaponifiable matter was chromatographed on 'Mayfair' alumina (Savory & Moore, Ltd.) and the chromatogram developed with 10% and thereafter with 15% benzene in light petroleum. The chromatogram formed a series of five zones below a small narrow brown 'oxidized' zone: (1) narrow red, (2) narrow red, (3) orange red, (4) light orange, (5) broad deep orange (typical carotene), in order of decreasing adsorption.

Zones 1 and 2 were combined, eluted with a mixture of ether, benzene and alcohol, and rechromatographed on 'Mayfair' alumina when lower zones containing carotenoids having the spectroscopic characteristics of lycopene (abs. max. at 543, 504, and 476 mμ. in carbon disulphide) and *neo*-lycopene (abs. max. at 535, 501·5, and 471 mμ. in carbon disulphide) were obtained.

Zone 3 on being rechromatographed furnished γ-carotene (abs. max. at 531, 497 and 465 mμ. in carbon disulphide and at 590 mμ. in the antimony trichloride reaction).

Zone 4 on being rechromatographed furnished *neo*-γ-carotene (abs. max. at 489 and 458·5 mμ. in carbon disulphide and at 591 mμ. in the antimony trichloride reaction).

Zone 5 was twice rechromatographed on 'Mayfair' alumina in order to obtain a satisfactory separation of α and β-carotene. The central portion of the β-carotene zone on elution furnished β-carotene which after recrystallization from benzene-acetone (1:20) had m.p. 183° and $E(1\%, 1\text{ cm.}) = 2240$ at 463 mμ. in benzene. The α-carotene zone furnished α-carotene which after recrystallization from benzene-acetone (1:40) had m.p. 186·5° and $E(1\%, 1\text{ cm.}) = 2330$ at 460 mμ. in benzene.

Abepa oil. The sample showed an extinction coefficient of 2·2 at 460 mμ. in benzene. A carotene estimation on 100 g. of the oil gave 0·0668% of carotene (α+β). The dextrorotation of the mixture in benzene was 177°, corresponding to 28% of α-isomer.

The petrol-soluble unsaponifiable matter obtained from 400 g. of the oil was chromatographed on 'Mayfair' alumina and the chromatogram was developed with 30% benzene in light petroleum which enabled the lower carotene zone to be separated as a percolate. The upper zones furnished carotenoids having the spectroscopic characteristics of lycopene (abs. max. at 545, 507, and 476 mμ.), *neo*-lycopene (abs. max. at 536·5, 501, and 474·5 mμ.), γ-carotene (abs. max. at 531, 497, and 468·5 mμ.) and *neo*-γ-carotene (abs. max. at 522, 490, and 460 mμ. in carbon disulphide).

The percolate on being rechromatographed furnished specimens of α- and β-carotene which after recrystallization had m.p. 184° (abs. max. at 511, 479, and 449·5 mμ. in carbon disulphide and $E(1\%, 1\text{ cm.}) = 2320$ in benzene) and 182° (abs. max. at 518, 487·5, and 457·5 mμ. in carbon disulphide and $E(1\%, 1\text{ cm.}) = 2290$ in benzene) respectively.

The residual soap solution from the petrol extraction was diluted with water and extracted with ether (2000 ml., 1500 ml., 1000 ml., 1000 ml.) and the combined extracts were washed, dried with anhydrous Na_2SO_4 and evaporated with the usual precautions. The ether-soluble unsaponifiable matter thus obtained was dissolved in a mixture of benzene-light petroleum (1:5) and chromatographed on a long column of partially deactivated 'Mayfair' alumina (300 g.) and the chromatogram was developed with 100 ml. portions of 20, 30, 50, and 75% benzene in light petroleum and finally with benzene. A series of six zones was obtained, (1) yellow, (2) orange, (3) canary yellow, (4) dull yellow, (5) salmon, (6) pink, in order of decreasing adsorption.

The material eluted from zone 2 was dissolved in hot carbon disulphide (3 ml.) and the solution was diluted with hot light petroleum (5 ml.), filtered, and kept in a refrigerator when lutein separated in orange crystals, m.p. 167° which showed absorption maxima at 507, 476, and 449 mμ. in carbon disulphide, at 486, 456·5, and 431 mμ. in chloroform and a band at 620 mμ. in the antimony trichloride reaction.

Lisombe oil. (i) The sample showed an extinction coefficient of 1·94 at 460 mμ. in benzene. A carotene estimation on 50 g. of oil gave 0·072% of carotene (α+β). The dextrorotation of the mixture was 299°, corresponding to 47% of α-isomer.

The petrol-soluble unsaponifiable matter from 500 g. of the oil was chromatographed and the chromatogram was developed with 30, 40, 50, and 60% benzene in light petroleum. The lycopene zone on being rechromatographed furnished a small quantity of solid lycopene which showed absorption maxima at 546·5, 506·5, and 478 mμ. in carbon disulphide. The γ-carotene zone furnished a small quantity of solid γ-carotene which showed absorption maxima at 530, 496, and 466 mμ. in carbon disulphide. The main carotene zone furnished α-carotene which had m.p. 183°, $E(1\%, 1\text{ cm.}) = 2340$, and $[\alpha]_D = +608°$ after recrystallization, and β-carotene which had m.p. 184° and $E(1\%, 1\text{ cm.}) = 2320$ after recrystallization. Spectroscopic evidence was obtained of the presence of *neo*-γ-carotene and *neo*-lycopene.

(ii) A second sample of *Lisombe* oil which had been prepared by the native method from the same collection of fruit was also examined. This sample showed an extinction coefficient of 3·13 at 460 mμ. in benzene. A carotene estimation on 100 g. of the oil gave 0·103% of carotene (α+β). The dextrorotation of the mixture was 222°, corresponding to 35% of α-isomer.

The petrol-soluble unsaponifiable matter from 450 g. of the oil was chromatographed on a large column of 'Mayfair' alumina. Zones were obtained which furnished small amounts of lycopene (m.p. 166°, abs. max. at 546·5, 507·5, and 475 mμ. in carbon disulphide) and γ-carotene (m.p. 148°, abs. max. at 531·5, 497, and 461 mμ. in carbon disulphide). The main carotene zone furnished α-carotene which had m.p. 186·5° and $E(1\%, 1\text{ cm.}) = 2380$ and β-carotene, m.p. 183° and $E(1\%, 1\text{ cm.}) = 2260$.

Spectroscopic evidence was obtained of the presence of *neo*-lycopene and *neo*-γ-carotene.

Commercial 'Sese' palm oil. The sample showed an extinction coefficient of 3·18 at 460 mμ. in benzene. A carotene estimation on 50 g. of oil gave 0·107% carotene (α+β). The dextrorotation of the mixture of isomers was 159°, corresponding to 25% of α-carotene.

The petrol-ether unsaponifiable matter from 1500 g. of oil finally furnished a specimen of lycopene, m.p. 172·5° which showed absorption maxima at 546, 507, and 477 mμ. in carbon disulphide and had $E(1\%, 1\text{ cm.}) = 2830$ in benzene and a specimen of γ-carotene, m.p. 143° which showed absorption maxima at 530·5, 497·5, and 464 mμ. in carbon disulphide and had $E(1\%, 1\text{ cm.}) = 2170$ in benzene. The main carotene zone yielded α-carotene, m.p. 186° of $E(1\%, 1\text{ cm.}) = 2370$ and β-carotene, m.p. 183° of $E(1\%, 1\text{ cm.}) = 2310$.

Spectroscopic evidence was obtained of the presence of *neo*-γ-carotene and *neo*-lycopene.

The ether-soluble unsaponifiable matter furnished a zone containing a carotenoid having the spectroscopic characteristics of *neo*-lutein (abs. max. at 501·5, 473, and 444 mμ. in carbon disulphide and at 620–30 mμ. in the antimony trichloride reaction).

SUMMARY

1. Examination of samples of 'Sese' palm oil obtained from specially selected bunches of fruit and of commercial samples of ordinary 'Sese' oil showed the presence of α, β, and γ-carotenes, lycopene, lutein and their *neo*-isomers.

2. In a single instance, evidence was obtained of the presence of a substantial amount of cryptoxanthin in a commercial sample of 'Sese' palm oil.

We thank the Directors of Lever Brothers and Unilever Ltd. for permission to publish these results.

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Lipotropic Factors and the Fatty Liver Produced by Feeding Cholesterol

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(*Received* 1 *May* 1946)

The excessive deposition of liver lipids which is produced in rats by feeding diets containing cholesterol is inhibited by choline and betaine (Best & Ridout, 1933; Best, Channon & Ridout, 1934). Choline was shown to affect both glycerides and cholesteryl esters but deposition of the former was inhibited more than that of the latter. Many preventive and curative experiments were subsequently reported and the results were in general agreement with those of the earlier investigations.

In the early studies no evidence of any significant waning in the lipotropic effect of choline was observed with or without cholesterol in the diet (Best & Ridout, 1936). Himsworth & Glynn (1944), however, report that this occurs in cholesterol-fed rabbits under certain experimental conditions. Furthermore, it has been stated by McHenry & Patterson (1944) that inositol but not choline has a definite effect on cholesterol metabolism. It became evident, therefore, that further studies were necessary to determine (*a*) the relative lipotropic effects of choline and inositol, and (*b*) the duration of the action of these agents when fed separately and in combination.

These problems have been reinvestigated in cholesterol-fed rats observed for periods up to 16 weeks. The effectiveness of choline in preventing deposition of cholesteryl esters did not diminish during this period, while that of inositol, which initially was definitely less than that of choline, progressively decreased. Under certain conditions inositol exerted no lipotropic action.

EXPERIMENTAL

White rats of the Wistar strain, reared in our own colony, were used. A few days before an experiment was started the animals were placed in individual cages, with a false bottom of coarse wire screen, in order to accustom them to the environment. The groups in each experiment were then balanced as far as possible with respect to weight and sex.

Weighed amounts of fresh diet were given daily, in feed trays designed to minimize spilling, and the following morning the amount left over and the scatter were weighed. From these data the individual daily food consumptions were calculated. The average values are recorded in the Tables. The rats were 'group pair-fed' and cared for as described previously (Best, Lucas, Patterson & Ridout, 1946).

The composition of the basal diets is given in Table 1. The supplements used in the test diets are shown in Tables 2–4. Choline was incorporated as its chloride, in molecularly equivalent amount, e.g. 0·345% choline chloride was used in diets stated to contain 0·30% choline.

In Exp. 1 the test diets (see Table 2) were fed for periods of 3, 8 and 16 weeks in order to determine whether any

Table 1. *Percentage composition of basal diets*

	Content (%) in diet nos.								
Component	1	1A	2	2A	3	3A	3B	3C	3D
Casein	8	8	8	8	10	10	10	10	10
Gelatin	12	12	12	12	—	—	—	—	—
Sucrose	60	59·5	73	70	84	81	51	51	51
Salts	5	5	5	5	4	4	4	4	4
Cellu flour	2	2	2	2	2	2	2	2	2
Beef dripping	10	10	0	0	0	0	30	—	20
Corn oil	2	2	0	2	0	0	—	30	10
Vitamin powder*	1†	1†	‡	‡	0	1	1	1	1
C.L.O. concentrate§	0·010	0·010	0·010	0·010	0·015	0·015	0·015	0·015	0·015
Cholesterol	0	0·5	0	1	0	2	2	2	2

* The vitamin powder consisted of aneurin hydrochloride 500 mg., riboflavin 250 mg., pyridoxin 200 mg., calcium pantothenate 1000 mg. and nicotinic acid 1000 mg. made to 1000 g. with very finely powdered (100 mesh) sucrose. Rats eating 10 g. of food per day receive 50 μg. aneurin hydrochloride and proportional amounts of the other vitamins daily.

† Diets 1 and 1 A contained not only the usual B vitamins but also per 10 g. diet, 1·5 mg. vitamin E (α-tocopheryl acetate) and 50 μg. vitamin K (2-methyl 1, 4-naphthoquinone).

‡ The rats received daily subcutaneous injections of the following B vitamins in 0·5 ml. physiological saline; aneurin hydrochloride 50 μg., riboflavin 25 μg., pyridoxin 20 μg., calcium pantothenate 100 μg. and nicotinic acid 100 μg.

§ The cod-liver oil concentrate (Ayerst, McKenna & Harrison, Ltd. Montreal), contained 200,000 i.u. vitamin A and 50,000 i.u. vitamin D/g.

waning of the lipotropic effect of choline or inositol occurred during this period. In Exp. 2, of duration 8 weeks, a comparison was made of the relative effectiveness of choline and inositol, singly and in combination, when fed at several different levels, in diets with and without added cholesterol (Table 3). The rats in Exp. 3 were fed a ration devoid of B vitamins for a preliminary period of 3 weeks. Comparable groups of these animals were then fed the cholesterol-containing test diets (Table 4) for 1 week to examine the effect of the preliminary period of vitamin depletion (similar to that used by McHenry and his associates) upon the relative efficacy of the lipotropic factors.

The animals were stunned and the livers removed immediately. The liver lipids were extracted with hot absolute ethanol and analyzed as described by Best *et al.* 1946.

RESULTS

The data for total lipids (Tables 2–4) are presented as percentage of wet liver weight for ease of comparison with many values in the literature. The individual components of the liver lipids are reported as mg./liver. From these values and the other data in the Tables, each component may be calculated to either a wet weight or fat-free dry residue weight basis. For many purposes the latter method of expression is to be preferred. The magnitude of normal values for liver lipids expressed as percentage of dry, fat-free residue weight, was determined to serve as a base for comparison. Livers of ten rats (125–160 g.) on a stock diet (Master fox breeder cubes), analyzed individually, gave: total lipids, average 25·3 (range 20·1–31·6); phospholipids 11·8 (10·2–12·9); free cholesterol 0·87 (0·73–0·98); total cholesterol 1·02 (0·90–1·21); cholesteryl esters, calculated as oleate 0·26 (0·18–0·36); glyceride 12·4 (8·8–17·7). A summary of the more pertinent data, calculated on the basis of fat-free dry residue, is given in Table 5.

DISCUSSION

Exp. 1. The results (Tables 2 and 5) provide further evidence that the lipotropic action of choline is not evanescent. They do not support the claim that inositol is more effective than choline upon cholesteryl esters.

Total liver lipids of rats on the cholesterol-free ration (diet 1, groups 1, 5 and 9) continued to increase beyond the three-week period frequently used in previous studies. The value obtained at 8 weeks was, however, just as high as at 16 weeks. As the glycerides accumulated in the liver, cholesteryl esters tended to rise proportionately, whether dietary cholesterol was present or not (cf. also groups 2, 6 and 10, in which cholesterol was present in the diet). Comparison of these two sets of data shows that the amount of cholesterol in the diet affects the ratio in which cholesteryl esters and glycerides are deposited. A graphic representation

Table 2. *Effects of dietary supplements on the rate of deposition and composition of liver lipids*

(Average initial weight of rats 150 g. (range 125–175 g.).)

							Liver								
		Addition to diet					Average wet weight (g.)	Average fat-free dry residue weight (g.)	Total lipids (% wet liver wt.)	Composition of liver lipids (mg./liver)					
Basal diet no.	Group no.	Substance	%	Survivors/ Starters	Average food intake (g./day)	Weight change (%)				Total lipids	Phospholipids	Cholesterol Free	Cholesterol Total	Cholesterol Ester*	Glyceride
Test diets fed for 3 weeks															
1	1	None	—	12/12	10·4	+ 2	10·4	1·748	27·19	2833	210	14·6	31·7	29	2579
1A	2	None	—	11/12	10·6	5	11·3	1·720	37·69	4248	248	20·9	147·7	214	3765
	3	Choline	0·3	10/12	11·5	12	8·5	1·779	8·38	712	178	16·5	89·6	123	394
	4	Inositol	0·3	11/12	10·9	3	11·1	1·651	30·35	3373	248	20·0	117·5	164	2941
Test diets fed for 8 weeks															
1	5	None	—	8/12	10·1	+ 3	13·3	1·837	37·50	4973	236	20·4	78·1	97	4619
1A	6	None	—	9/12	10·6	9	16·2	2·075	37·59	6089	280	37·7	412·1	631	5141
	7	Choline	0·3	10/12	9·9	27	7·8	1·748	8·08	627	236	16·8	91·7	126	248
	8	Inositol	0·3	7/12	10·0	6	13·4	1·825	39·31	5246	270	33·6	273·9	405	4538
Test diets fed for 16 weeks															
1	9	None	—	10/12	10·0	+15	14·6	2·042	36·68	5420	247	23·5	78·5	93	5057
1A	10	None	—	9/12	10·0	8	16·7	2·051	38·66	6463	230	44·5	490·3	753	5436
	11	Choline	0·3	9/12	10·4	54	8·7	2·101	7·71	672	253	18·8	73·7	92	308
	12	Inositol	0·3	8/12	10·3	33	14·9	2·042	36·29	5418	246	34·4	400·7	617	4521

In this and subsequent Tables, the cholesteryl ester value has been expressed as cholesteryl oleate, obtained by multiplying the weight of bound cholesterol by the factor 1·684.

Table 3. *Lipotropic effects of choline and inositol with and without cholesterol*

Basal diet no.	Group no.	Addition to diet: Substance	Addition to diet: %	Survivors/ Starters	Average food intake (g./day)	Weight change (%)	Liver: Average wet weight (g.)	Liver: Average fat-free dry residue weight (g.)	Liver: Total lipids (% wet liver wt.)	Composition of liver lipids (mg./liver): Total lipids	Phospholipids	Cholesterol: Free	Cholesterol: Total	Cholesterol: Ester*	Glyceride
2*	13	None	—	21/22	11·5	0	10·5	1·584	31·24	3238	240	18·6	77·7	100	2879
	14	Choline	0·1	9/10	11·4	+14	7·9	1·792	8·30	659	266	13·8	26·1	21	359
	15	Choline	0·5	12/12	11·3	+ 9	7·0	1·613	8·26	582	273	15·0	24·6	16	278
	16	Inositol	0·1	9/10	10·6	+ 4	8·0	1·442	21·81	1744	228	14·8	38·9	41	1461
	17	Inositol	0·3	11/12	11·4	− 4	8·7	1·615	23·34	2084	264	17·6	41·6	40	1762
	18	Choline + inositol	0·1 + 0·1	9/10	11·5	+19	6·7	1·510	7·18	483	249	13·3	21·7	14	208
	19	Choline + inositol	0·1 + 0·3	10/10	10·9	+17	7·0	1·617	6·44	453	239	12·8	18·8	10	191
	20	Choline + inositol	0·5 + 0·3	11/12	11·7	+10	6·7	1·615	6·20	415	252	15·0	18·2	5	142
2A*	21	None	—	20/22	11·3	+ 4	12·1	1·591	39·71	4824	283	28·9	316·7	485	4027
	22	Choline	0·1	10/10	10·8	+26	7·0	1·493	15·00	1056	280	16·5	112·5	162	597
	23	Choline	0·5	11/12	11·4	+ 6	6·2	1·454	8·95	556	241	16·4	56·8	68	231
	24	Inositol	0·1	10/10	10·8	+18	11·2	1·356	34·86	3915	265	23·0	250·0	382	3245
	25	Inositol	0·3	11/12	11·1	+ 1	11·4	1·523	36·04	4109	275	26·4	231·8	346	3462
	26	Choline + inositol	0·1 + 0·1	10/10	11·2	+25	6·6	1·481	12·20	808	271	17·0	118·9	168	352
	27	Choline + inositol	0·1 + 0·3	9/10	10·5	+21	6·0	1·375	10·28	620	241	14·3	58·3	74	290
	28	Choline + inositol	0·5 + 0·3	11/12	11·9	+11	6·5	1·555	7·52	485	275	16·4	30·7	24	169

* Limited availability of animals necessitated doing the experiment in two sections with a time interval of about a month. The average weight of the 12 animals used in each group in the first part was 175 g. (range 140–220 g.) and of the 10 in the second part was 140 g. (range 80–200 g.). The difference in size of rats used in the two parts of the experiment did not affect appreciably the total liver lipids (the values in the two basal groups were: diet 2, 33·11 and 28·74, diet 2A, 38·83 and 40·78% of wet liver weight) or the composition of the lipids. The figures given in the Table for basal Groups 13 and 21 are weighted averages of the two sets of values.

of another example of this relationship has been published recently (Best *et al.* 1946).

When the basal diet contained cholesterol (groups 2, 6 and 10) the liver lipids appeared to reach a maximum within 3 weeks, if calculated as percentage of wet liver weight. If expressed as percentage of fat-free dry residue weight, however, it will be noted (Table 5) that the liver lipids continued to increase throughout the test period, the values at 3, 8 and 16 weeks being 247, 294 and 315%, respectively. This presumably means that, after 3 weeks, fat and water tend to be deposited in the same ratio. The increase in fat was due mainly to accumulation of glycerides with smaller increments due to cholesteryl esters.

The inclusion of 0·3% choline (groups 3, 7 and 11) in the diet kept the total liver lipids at a low level (about 8% of wet liver weight, which is only slightly above normal) throughout the whole 16-week period.* Inositol fed at the same level (groups 4, 8 and 12) was never nearly as effective as choline. At 3 weeks choline had reduced total lipids from 247 to 40% of fat-free dry residue weight, while inositol had brought the value down only to 204%, and at 16 weeks the influence of inositol was even less (Table 5).

Free cholesterol showed a small and possibly insignificant rise in the livers of the animals on the cholesterol-free ration (diet 1, groups 1, 5 and 9) as the experiment was prolonged (Table 5). The presence of 0·5% cholesterol (diet 1A, groups 2, 6 and 10) caused a slightly greater and probably significant increase in the free sterol. Chanutin & Ludewig (1933) have also noted increases in free cholesterol after feeding cholesterol under somewhat different conditions. Choline tended to restore the level of free cholesterol in the liver to normal (Table 5), while inositol had a negligible effect.

The cholesterol intake (0·5%), while considerably smaller than in some previous experiments, was still greatly in excess of the amount contained in the normal diet of rats. While cholesteryl esters were not reduced to normal values by 0·3% choline there was a marked and progressive reduction,

* Himsworth & Glynn (1944) noted an evanescent effect of 4 mg. choline daily in rats fed a diet containing 50% lard. Data to be published shortly indicate that this amount of choline is insufficient to maintain liver fat at low levels under these conditions.

Table 4. *Effect of preliminary depletion of B vitamins on lipotropic activity*

(Average initial weight of rats at beginning of test period 65 g. (range 55–85 g.).)

Basal diet no.	Group no.	Addition to diet: Substance	Addition to diet: %	Survivors/ Starters	Average food intake (g./day)	Weight change (%)	Liver: Average wet weight (g.)	Liver: Average fat-free dry residue weight (g.)	Liver: Total lipids (% wet liver wt.)	Composition of liver lipids (mg./liver): Total lipids	Phospholipids	Cholesterol: Free	Cholesterol: Total	Cholesterol: Ester*	Glyceride
3	29	None (Vit. B depletion)	—	6/6	7·2	−13	4·9	1·226	4·83	238	151	7·9	8·2	1	79
3A	30	None	—	12/12	10·2	+18	8·1	1·760	20·93	1701	199	15·4	50·0	58	1428
	31	Choline	0·5	12/12	10·2	+18	6·2	1·308	12·63	787	178	13·6	35·0	36	559
	32	Inositol	0·5	12/12	10·2	+20	6·3	1·280	15·78	998	178	12·5	36·5	41	766
3B	33	None	—	9/10	5·4	+ 5	6·1	0·833	32·43	1971	128	13·7	83·3	117	1712
	34	Choline	0·5	9/10	5·8	+18	4·5	0·952	10·95	490	123	11·4	30·7	33	324
	35	Inositol	0·5	9/10	5·3	+ 8	5·4	0·785	28·33	1523	117	12·6	76·4	107	1286
	36	Choline + inositol	0·5 0·5	10/10	5·9	+18	4·8	1·042	7·28	350	116	11·9	20·3	14	209
3C	37	None	—	8/10	5·0	+ 9	6·8	1·013	24·47	1670	131	12·3	80·7	115	1411
	38	Choline	0·5	9/10	5·3	+21	5·1	1·086	16·40	769	163	13·5	34·7	36	557
	39	Inositol	0·5	8/10	5·3	+13	6·2	0·912	30·96	1931	118	13·8	87·2	124	1675
	40	Choline + inositol	0·5 0·5	8/10	6·0	+22	5·1	1·133	8·71	449	118	10·9	35·9	42	278
3D	41	None	—	11/12	7·2	+ 6	7·4	1·076	35·96	2663	184	16·4	97·0	136	2327
	42	Choline	0·5	12/12	7·1	+ 7	4·6	1·092	8·32	386	173	12·0	43·1	53	148
	43	Inositol	0·5	12/12	7·0	+ 7	6·4	1·051	31·61	2088	173	14·8	106·3	154	1745

compared with the controls, throughout the 16-week period; at 3 weeks 0·3 % inositol was not as effective in reducing steryl esters as was choline and its effectiveness diminished as the experiment was prolonged.

The constancy of the phospholipid values (12·0, 11·3, 12·0, 12·0 % fat-free, dry residue weight for Groups 9, 10, 11 and 12, respectively) is remarkable in view of the large changes in glyceride and cholesteryl esters.

Exp. 2. The results of this experiment (Table 3), in which choline and inositol were fed singly and in combination, at several different levels, for 8 weeks confirm and extend the above-mentioned findings. The first comparisons were made using fat-free test diets (diet 2, groups 13–20) and a similar study was made with diets containing 2 % corn oil (Mazola) plus 1 % cholesterol (diet 2A, groups 21–28).

On the fat-free diets choline fed at the 0·1 % level was almost as effective on all the lipid components as when 0·5 % was provided (compare groups 14 and 15, Table 5). In the presence of cholesterol (groups 22 and 23) the smaller dose of choline was inferior to the larger. The latter brought the liver glycerides nearly to normal and caused an 85 % reduction in cholesteryl esters. Therefore even large amounts of dietary cholesterol (1 %) do not produce a condition as unfavourable for the action of choline as one might be led to expect from statements in the literature (McHenry & Patterson, 1944).

Inositol at 0·1 % dietary level exerted only a very limited lipotropic effect in the rats on the fat-free diet (Group 16) and even less in the presence of the corn oil plus cholesterol (Group 24). Three times as large an intake of inositol had no significantly greater effect on the glycerides and produced only a slightly greater decrease in cholesteryl esters.

In Tables 3 and 5 the effects of various combinations of choline and inositol are also presented. Inositol (0·1 %) added to the diet free from fat and cholesterol (diet 2) did not augment the effect of 0·1 % choline on total lipids or glycerides (compare groups 14 and 18, Table 5). The slight decrease in cholesteryl esters is of questionable significance. When the amount of inositol was increased to 0·3 % a slight but definite effect on both glycerides and steryl esters was noted (groups 14 and 19). Addition of 0·3 % of inositol to a diet already containing 0·5 % choline brought both glycerides and cholesteryl esters within the normal range (groups 15 and 20).

In diets containing fat and cholesterol (groups 21–28), the combined effects of choline and inositol in the amounts mentioned above were qualitatively

Table 5. *Composition of liver lipids*

Basa diet	Group no.	Supplement	%	Duration of exp. (days)	Liver: Total lipid	Liver: Free cholesterol	Liver: Cholesteryl ester	Liver: Glyceride
					% fat-free, dry residue weight			
1	1	None	—	21	162	0·83	1·7	148
1A	2	None	—	21	247	1·21	12·4	219
	3	Choline	0·3	21	40	0·93	6·9	22
	4	Inositol	0·3	21	204	1·21	9·9	178
1	5	None	—	56	271	1·10	5·3	252
1A	6	None	—	56	294	1·82	30·4	248
	7	Choline	0·3	56	36	0·96	7·2	14
	8	Inositol	0·3	56	288	1·84	22·2	249
1	9	None	—	112	265	1·15	4·5	247
1A	10	None	—	112	315	2·17	36·7	265
	11	Choline	0·3	112	32	0·90	4·4	15
	12	Inositol	0·3	112	265	1·69	30·2	221
2	13	None	—	56	218	1·17	6·3	195
	14	Choline	0·1	56	52	0·78	1·2	35
	15	Choline	0·5	56	36	0·94	1·0	17
	16	Inositol	0·1	56	168	1·02	2·8	148
	17	Inositol	0·3	56	137	1·18	2·7	115
	18	Choline 0·1 + inositol 0·1		56	53	0·88	0·9	35
	19	Choline 0·1 + inositol 0·3		56	40	0·79	0·6	24
	20	Choline 0·5 + inositol 0·3		56	26	0·93	0·3	9
2A	21	None	—	56	301	1·82	30·6	252
	22	Choline	0·1	56	71	1·11	10·8	40
	23	Choline	0·5	56	38	1·13	4·7	16
	24	Inositol	0·1	56	289	1·68	28·2	239
	25	Inositol	0·3	56	270	1·73	22·7	227
	26	Chloine 0·1 + inositol 0·1		56	55	1·15	11·4	24
	27	Choline 0·1 + inositol 0·3		56	45	1·04	5·4	21
	28	Choline 0·5 + inositol 0·3		56	31	1·05	1·6	11
3	29	None (Vit. B depletion)	—	21	19	0·65	0·05	6
3A	30	None	—	7	97	0·88	3·3	81
	31	Choline	0·5	7	60	1·04	2·8	43
	32	Inositol	0·5	7	78	0·98	3·2	60
3B	33	None	—	7	237	1·64	14·1	205
	34	Choline	0·5	7	52	1·20	3·4	34
	35	Inositol	0·5	7	194	1·60	13·7	164
	36	Choline 0·5 + inositol 0·5		7	34	1·14	1·4	20
3C	37	None	—	7	165	1·21	11·4	139
	38	Choline	0·5	7	71	1·25	3·3	51
	39	Inositol	0·5	7	212	1·51	13·6	184
	40	Choline 0·5 + inositol 0·5		7	40	0·97	3·7	25
3D	41	None	—	7	248	1·52	12·6	216
	42	Choline	0·5	7	35	1·10	4·8	14
	43	Inositol	0·5	7	199	1·40	14·6	166

similar to those described in the preceding paragraph. The level of steryl esters in the basal group (21) was increased five-fold by feeding cholesterol. A profound decrease was produced by 0·1% choline. This effect was not augmented by 0·1% inositol but was by 0·3%. The level of steryl esters was lowered still further when choline 0·5% and inositol 0·3% were supplied (group 28) but the value remained above normal. Thus the synergism of choline and inositol can be clearly demonstrated

at certain dosage levels, but here again there is no evidence of any preferential effect of inositol on cholesteryl esters.

Exp. 3. This experiment was designed to compare the relative effectiveness of choline and inositol after a preliminary three-week period of depletion of B-vitamins (the procedure followed by McHenry and his associates). The influence of dietary fat upon the liver lipids of cholesterol-fed animals was also determined. Diet 3A contained 2% cholesterol without any fat, 3B contained an essentially saturated fat (beef dripping 30%), 3C contained the same amount of an unsaturated oil (Mazola) and 3D contained a mixture of these two fats (beef dripping 20%, Mazola 10%).

During the depletion period (diet 3), the food consumption fell from about 8 g. per day to about 3 g. The animals lost weight and several died. The survivors were divided into comparable groups to which the test diets were fed for 1 week only.

In every case choline was more effective than inositol in reducing total liver lipids and cholesteryl esters. The presence of fat in the basal diets (3B, 3C and 3D) increased the total liver lipids greatly. It appears to have caused a slight rise in the free cholesterol (Table 5) and about a four-fold increase in the steryl esters (compare group 30 with 33, 37, 41). The nature of the dietary fat influenced the deposition of glycerides in rats on the basal diets and, to a much smaller extent, that of steryl esters (compare groups 33 and 37). The slightly lower cholesteryl ester value in the rats receiving the corn oil (group 37) as compared with those on beef dripping (group 33) is probably a reflexion of the lower glyceride content of the livers of the 'corn-oil group'. It would appear from the data for groups 34 and 38 that choline is somewhat more effective in reducing glycerides in animals on beef dripping than it is when corn oil is the dietary fat. Inositol produced a small decrease in glycerides in rats on the beef dripping, but seemed to increase the deposition of glycerides in the 'corn-oil group'. This effect, which requires further study, has been noted previously (Beveridge & Lucas, 1945; Handler, 1946). The synergistic effect of choline and inositol was again evident.

When the two fats were present (Basal Diet 3D) inositol had some effect on glycerides, in contrast to the lack of effect on the 'corn-oil diet' (3C) (cf. groups 37, 39 with 41, 43). However, there was no lipotropic effect of inositol on steryl esters. In the presence of beef dripping there appears to be a partial removal of the blockage of the lipotropic effect of inositol which is observed in the diet containing corn oil alone. An almost completely saturated fat-fraction from beef dripping and a highly unsaturated one from corn oil, have been prepared and their influence upon the lipotropic activity of inositol is being studied.

SUMMARY

1. No waning of the lipotropic effect of choline was observed in rats fed a diet containing 0·5% cholesterol for 16 weeks.

2. Inositol, fed at the same level as choline, was never nearly as active in decreasing glycerides or cholesteryl esters and its effectiveness diminished as the experiment was prolonged.

3. The synergistic lipotropic effect of choline plus inositol was clearly demonstrated in cholesterol-fed rats but inositol did not exert any preferential effect on cholesteryl esters.

4. In experiments, similar to those of McHenry & Patterson (1944) and of Handler (1946) in which the rats were given a preliminary period of deficiency of vitamin B_1, choline was uniformly more effective than inositol in decreasing liver glycerides and cholesteryl esters in cholesterol-fed rats receiving diets with or without fat. These results agree with those reported by Handler (1946).

5. Further evidence that the nature of the dietary fat affects the lipotropic action of inositol has been presented and briefly discussed.

We are indebted to our colleague, Dr C. S. McArthur, whose improvements in the Schoenheimer-Sperry procedure for determining cholesterol made it applicable to tissues containing abnormally large amounts of fat. The expenses of this investigation were defrayed, in part, by a grant from the Banting Research Foundation.

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Vitamin A in the Horse

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(*Received* 12 *April* 1946)

Comparatively little work has been done on the metabolism of vitamin A in the horse. The diet of this animal, although adequate in carotene in farms, may be poor in this respect under urban conditions. It is perhaps surprising therefore that so many horses should appear well and suffer little from disabilities while subsisting on diets low in carotene. There is ample evidence, however, that severe deficiency eventually causes illness. Klemola (1933) found changes in the hoofs of army horses on a ration of oats and hay. Rapid improvements were effected by grazing, or by administering A.I.V. silage (Virtanen, 1933) and cod-liver oil. Vitamin A deficiency in army horses on a ration of straw and hay was also reported by Chatelain (1933) and later by Kesler & Callender (1939). Mitchell (1936) considered that, while definite proof of deficiency in the horse was lacking, some of the commoner diseases, such as rheumatism and the high percentage of abortions in the mare, might be due to vitamin A deficiency. Edwards (1937–8) supported this hypothesis. Skackov (1940), on the other hand, found that neither carrots nor cod-liver oil had any effect on abortions in mares, while others denied that joint lesions were due to vitamin A deficiency (Hart, Goss & Guilbert, 1943). In chemical estimations of vitamin A in horse's liver Linton (quoted by Mitchell, 1936) found little or no vitamin, although Jensen & With (1939) found about 500 i.u./g. Cruickshank (1945) found no carotene in the hay etc. on which pit ponies in a certain mine were supposed to subsist, although they were healthy in appearance and worked hard in the dark. In the absence of some crucial test, however, there is no real proof that they were not night blind.

Experimental vitamin A deficiency has been produced in horses by Guilbert, Howell & Hart (1940). They observed night blindness, roughness of the skin and other symptoms of avitaminosis, but no keratinization of the cornea was seen. The untreated animals eventually died. Andersen & Hart (1943) found that one horse on vitamin A-free diet developed night blindness in 502 days, and when it was killed after another 152 days no vitamin A was found in the liver. A control animal at the same time had 1173 i.u./g. of liver.

EXPERIMENTAL

In the present study, the vitamin A content of the serum and liver of horses has been investigated. Serum was collected from 7 horses belonging to the Institute of Animal Pathology, University of Cambridge. These animals had been kept on a diet of chaff, bran, oats and hay, and had received no green stuff for over 12 months. They appeared normal, healthy, lively and smooth coated. In the eyes there was no noticeable abnormality such as excessive lachrymation or discharges. Other samples of serum and of liver were collected from farm horses, 2–10 years old, at a knacker's yard. These animals had been killed not on account of disease, but because they were more valuable as carcasses than alive. They had been in normal health, and had been passed by qualified veterinary surgeons as fit for human consumption. Most of these horses had probably received considerable amounts of carotenoids by grazing. Serum vitamin A was estimated by the method of Yudkin (1941). Liver assays were made by the method of Davies (1933) with modifications introduced by Moore (1937). Values for serum vitamin A have been corrected for the presence of carotenoids. The small proportion of carotenoids in the liver has been neglected in calculating the vitamin A reserves. The results are given in Table 1.

Table 1. *Vitamin A, carotenoids, and bilirubin in the serum and liver of the horse*

	Serum			
No. of horse	Total carotenoid (i.u./100 ml.)	Vitamin A (i.u./100 ml.)	Bilirubin (mg./100 ml.)	Liver Vitamin A (i.u./g.)
1	12	32	1·0	—
2	25	7	1·6	—
3	14	27	1·4	—
4	14	28	1·3	—
5	8	31	2·1	—
6	6	17	2·2	—
7	6	32	1·2	—
8	—	—	—	1200
9	48	10	3·5	630
10	95	38	2·6	540
11	6	20	2·6	630
12	48	32	4·9	48
13	8	49	3·7	78
14	42	52	4·4	430
15	75	52	7·4	2000
16	0	50	3·3	171
17	390	6	3·9	1500
18	35	23	4·4	735
19	12	29	2·1	189
20	36	23	3·3	720
21	8	44	2·8	610
22	0	31	2·5	32
23	6	30	2·7	780
24	60	37	4·3	480
25	34	24	4·5	375
26	146	15	3·7	400
27	288	9	1·3	375
28	220	15	4·4	400
29	442	22	5·0	1500
30	34	40	1·7	—
Means	73	28	3·1	628

It is evident that the vitamin A content of the serum of horses is usually of a low order. Clearly even when the animals have a large intake of carotenoids, as evidenced by a high vitamin A reserve in the liver, or by a high value for serum carotenoids, the vitamin A content of the serum remains low. Thus, although horse 15 had 2000 i.u. of vitamin A/g. of liver, and no. 29 had 1500 i.u. of vitamin A/g. of liver and 442 'i.u.' of carotenoids/100 ml. of serum, the highest serum vitamin A for any horse was only 52 i.u./100 ml. Too much reliance, however, should not be placed in the very low values found in some horses having high serum carotenoids, in view of the uncertainty of the correction factor for carotenoids in the $SbCl_3$ reaction. Little correlation can be traced between either the plasma carotenoids and vitamin A, or between the vitamin A contents of the plasma and liver.

A surprising finding is that the serum of all the horses, without exception, contained relatively large amounts of bilirubin (Table 1) as estimated by the modified method of the author (Rudra, 1945). It is interesting to note that high vitamin A reserves in the liver generally synchronized with a high bilirubin content in the serum. The horse with the highest liver vitamin A (2000 i.u./g.) had also the highest serum bilirubin content (7·4 mg./100 ml.). It may also be significant that the farm horses, which presumably received much carotene in their diet, had also high bilirubin contents in their serum. No bilirubin could be detected in normal or vitamin A deficient calf serum, normal sheep serum or donkey serum. Royer, Chiaravalle & Aramburu (1941) also found that the blood plasma of the horse contained 0·5–3·0 mg. of bilirubin/100 ml., while a similar conclusion has recently been reached by Ramsay (1945). In the absence of evidence to the contrary we should regard a high serum bilirubin as a normal occurrence in this animal. The frequent presence of large amounts of bilirubin, in the serum of farm horses, and the lower range of values in horses living on a diet almost free from carotene, suggests some curious and novel relationships in the vitamin A metabolism in the horse, but no satisfactory explanation can yet be suggested.

SUMMARY

1. The vitamin A content of the serum of English horses varied from 6 to 52 i.u./100 ml. with a mean of 28 i.u. The vitamin A content of liver varied from 32 to 2000 i.u./g. with a mean of 628.

2. Horse serum normally contains bilirubin. The amounts found varied from 1·0 to 7·4 mg./100 ml., with a mean value of 3·1 mg.

The above work was carried out during the tenure of a Ghosh Travelling Fellowship of the University of Calcutta. The author is indebted to the Dunn Nutritional Laboratory, Cambridge, for hospitality, to Dr T. Moore for advice, and to Dr L. J. Harris for criticism.

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The Mineral Constituents of Bone

3. THE EFFECT OF PROLONGED PARATHORMONE INJECTIONS ON THE COMPOSITION OF THE BONES OF PUPPIES WITH VARYING CALCIUM INTAKES

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(*Received* 16 *January* 1946)

'Osteitis fibrosa cystica' has frequently been correlated with hyperparathyroidism in human beings (Hunter & Turnbull, 1931–2). Many workers have attempted to produce the condition in animals by a combination of repeated injections of parathyroid extract and other treatment such as diet. Bauer, Aub & Albright (1929) used cats, rats and rabbits, Bodansky, Blair & Jaffe (1929–30) guinea-pigs, Selye (1932) rats, Greenwald & Gross (1926), Jaffe & Bodansky (1930), Johnson (1932) and others used dogs. Herbivora needed very large doses of the hormone to produce a measurable effect on blood

composition or bone structure. In dogs, Jaffe & Bodansky (1930) and Johnson (1932) succeeded in reproducing the typical symptoms of the disease—bone fractures or deformities, and histological evidence of excessive bone resorption and repair, and invasion of the marrow cavity by fibrous tissue, with formation of multiple cysts. Jaffe & Bodansky (1930) gave their dogs lean meat, cod-liver oil, tomato juice and occasionally calcium salts; Johnson (1932) added bread to this. With increasing dosage of extract their dogs showed symptoms of overdosage and treatment had to be suspended temporarily. The bone changes were not found in uninjected dogs on the same diet. These workers concluded that the hormone brought about decalcification of bone. Bauer *et al.* (1929) concluded that this decalcification began in the trabeculae. None of these workers produced chemical evidence of decalcification of bone. The object of the experiments described here was to determine whether poorly calcified bone, or alternatively diminished bone growth relative to body growth, could be demonstrated by chemical analysis in dogs given injections of parathyroid extract over prolonged periods, where the dogs remained healthy throughout the experiment, and all phenomena of overdosage were avoided. The number of units injected was increased gradually, and their effect noted at three levels of calcium intake.

EXPERIMENTAL

Diet and treatment of animals. Three litters were used. Litter 1 had the 'good' diet, consisting of ½ pint of milk daily, meat with bones, fish heads, cod-liver oil and marmite, with porridge, potatoes and bread *ad libitum* for 120 days. Litter 2 on the 'moderate' diet received the same without bones or fish heads, and with a ¼ pint of milk daily for 100 days. Litter 3 was fed for 90 days on the same diet as litter 2, without milk, but with orange juice. Parathyroid extract was injected intraperitoneally on alternate days in litters 1 and 2, and daily in litter 3. Of the three in litter 1, one injected pup died at 107 days, the other with its control was killed at 170 days, having received 1500 units over 120 days. The six pups of litter 2 were killed at about 140 days, three of them having received 1500 units over 100 days. The four of litter 3 were killed at about 140 days, two having received 1300 units over 20 days. The preparation used for the first litter was Paratyrone (prepared by the Etablissements Byla, A. Conar et Panau, France), for the other two, Euparatone (Allen and Hanbury Ltd.). One pup of the first litter died after a single injection of Parathormone (Eli Lilly and Co. Ltd.) Collip's extract, after receiving injections of the Paratyrone preparation over 60–70 days.

Methods. Methods of sampling and analysis of the tissues have already been described (Burns & Henderson, 1935).

RESULTS

Effects of the diet

These are seen in Tables 1–3. The average increase in weight between the ages of 50 and 140 days was 4·3, 3·9 and 3·3 kg. in litters 1, 2 and 3 respectively. Bone length is unaffected by diet (Table 1, col. 13). In litter 3, wet bone weight is about 25 % less, dry fat-free bone about 33 % less, and the proportion of ash in dry fat-free bone about 10 % less than in litter 2 (Table 1, cols. 5, 6, 11). The rapidly growing cortex ends are equally well calcified in both litters, but the slower growing tissues in litter 2 are slightly better calcified than in litter 3 (Table 2). Thus the litter on the diet poor in calcium produced smaller bones of smaller calcium content. The poorer calcification of litter 3 is still marked if proportion of bone weight or ash to body weight is considered (Table 1, cols. 7, 10). The bones of litter 3 were definitely crumbly in texture compared with those of 2, the difference being much greater than might be expected from their respective ash contents.

The two animals aged 170 days of litter 1 had larger bones of higher ash content than those of litter 2, but not a greater proportion of bone to body weight (Table 1, cols. 5, 7). However, Table 2 shows that the cancellous and cortex (middle) parts of the 107-day-old femur were more calcified than those of the 140-day pups. In the humerus and tibia of this animal this was also true of the cortex ends. Therefore part of the higher degree of calcification in the 170-day pups is due to diet and not merely to age. It is more interesting that the serum calcium should vary with the calcium intake (Table 3), since it has not been shown to do so in dogs, though it does in rats (Greenberg & Miller, 1941).

Effects of parathyroid extracts

The difference in bone weight between the injected and control animal of litter 1 was not greater than variations among controls in litter 2, but bone weight, and ash, relative to body weight, was greater in the injected than in the control animal (Table 1, cols. 7, 10). The tibia of the injected animal bulged at the knee end. This was due to an overgrowth of fibrous bone on the tibial tuberosity. This tissue had a calcium content of 16·8 %. The dry weight of the overgrowth was about 25 % of that of the rest of the tibia 'cortex end', which had a calcium content of 20·5 %. This bulge was the only deformity noted in any animal. The low calcium content of the cortex end of 107-day femur (Table 2) may indicate a localized failure to calcify, but no sign of general failure in building or calcifying bone was found in injected animals of litter 1, nor equally in litter 3.

In litter 2 the only positive difference between injected and uninjected animals was an increase in water content and decrease in fat content in the bones of injected animals (Table 4). This might indicate an increase in the marrow cavity with the formation of more fibrous, less fatty marrow. The

Table 1. *Body weight, bone weight and weight and composition of dry, fat-free femur, tibia and humerus of dogs treated with parathyroid extract*

Column ...	1	2			3	4	5	6	7	8	9	10		11	12		13
							Wt. of 3 bones (g.)										
Litter and diet		Animal			Approx. age (days)	Body wt. at death (kg.) W	Wet	Dry fat-free B	$\left(\frac{\text{wt. bones}}{\text{wt. body}}\right) \times 10^4$ $B/W \times 10^4$	Average non-fatty solids in wet bone (%)	Average wt./length (g./cm.)	$\left(\frac{\text{wt. ash one bone}}{\text{wt. body}}\right) \times 10^5$		Ash in one dry fat-free bone (%)	Cancellous as percentage of wt. of femur		Femur length (cm.)
1, High Ca diet		Injected			107	3·5	49·0	23·2	66	47·4	0·90	—		—	14·0		9·2
		Injected			170	8·8 7·0*	90·4	46·1	52	51·2	1·27	102	Femur	54·3	17·8		12·5
		Control			170	8·0 5·8*	69·1	36·3	45	52·4	1·05	84	Femur	54·5	14·6		11·7
2, Moderate Ca diet		Squeaker	In-jected		140	5·2	72·1	25·4	48	35·3	0·84	70	Humerus	43·8	11·1	Knee only	10·5
		Fanny	In-jected			6·6	77·9	31·5	47	41·1	0·93	74	Humerus	50·2	13·6	Knee only	11·8
		Brown and White	In-jected			6·1	77·9	32·0	53	41·8	0·92	85	Humerus	47·7	12·1	Knee only	12·0
		Tiny	Con-trols			4·5	48·9	18·3	41	37·7	0·67	63	Humerus	45·6	10·7	Knee only	9·2
		Patches	Con-trols			8·8	92·5	39·8	45	43·3	1·15	75	Humerus	50·2	10·1	Knee only	12·0
		Brown and White	Con-trols			6·7	73·3	33·6	50	45·9	0·94	77	Humerus	49·8	10·5	Knee only	12·0
3, Low Ca diet		White ♂	In-jected			5·8	61·8	22·8	39	36·9	0·66	54	Humerus	45·6	17·4		11·7
		Black	In-jected			5·9	61·7	20·3	34	33·0	0·60	47	Humerus	41·1	20·1		11·7
		Brown	Con-trols			5·1	51·0	16·9	33	33·3	0·50	39	Humerus	40·0	14·6		10·7
		White ♀	Con-trols			4·3	48·6	19·1	45	37·5	0·60	60	Humerus	44·1	17·7		10·7

* At 140 days

Table 2. *Composition of mineral matter of bones*

Litter and diet	Approx. age (days)	Treatment	Animal	Ca (%)				Ca as carbonate (%)				P (%)			
				Calcified cartilage	Cancellous*	Cortex middle	Cortex end (knee)	Calcified cartilage	Cancellous*	Cortex middle	Cortex end (knee)	Calcified cartilage	Cancellous*	Cortex middle	Cortex end (knee)
1, High Ca	107	Injected		—	20·0	22·3	13·1†	—	8·7	10·9	10·4†	—	9·7	10·2	6·5†
	170	Injected		19·5	21·5	23·8	18·2	7·9	9·8	12·3	9·6	9·4	10·6	11·5	8·6
		Control		19·2	22·5	24·5	17·7	9·0	10·2	13·0	9·7	9·3	10·7	11·8	8·5
2, Moderate Ca	140	Injected	Squeaker	—	16·4	20·6	13·5	—	9·0	10·2	10·0	—	8·4	9·9	6·5
					16·7				8·6				8·4		
			Fanny	—	17·7	21·5	15·1	—	7·9	9·6	8·5	—	9·1	10·6	7·2
					19·0				7·3				9·5		
			Brown and White	—	18·4	20·6	15·6	—	10·1	11·4	11·0	—	9·2	10·3	7·2
					18·9				10·3				9·2		
		Control	Tiny	—	17·1	19·5	12·5	—	8·5	9·7	7·8	—	8·8	9·9	6·3
					17·0				8·4				8·8		
			Patches	—	18·5	21·4	15·5	—	7·8	9·6	9·4	—	9·7	10·7	7·6
					17·8				7·6				9·5		
			Brown and White	—	18·0	21·4	15·9	—	8·3	10·3	7·8	—	9·1	10·8	7·6
					18·2				8·6				9·2		
3, Low Ca	140	Injected	White ♂	17·7	17·1†	19·6	14·9	6·8	7·2†	9·6	7·8	9·9	9·5†	10·7	7·2
			Black	16·5	16·2	19·5	12·4	6·3	6·6	8·0	8·5	8·5	8·7	9·8	6·2
					15·8				6·5				8·2		
		Control	Brown	16·4	17·5	19·4	14·3	6·5	8·2	9·8	9·3	9·0	9·2	10·0	6·8
					17·2				6·8				8·7		
			White ♀	16·2	15·7	19·9	15·1	7·0	7·6	9·3	8·4	8·5	8·3	10·3	7·4
					15·7				7·1				8·4		

* Upper figure, hip; lower, knee end.

† Both ends.

Table 3. *Concentration of calcium in serum and kidney of dogs treated with parathyroid extracts*

Litter and diet	Animal		Calcium content (mg./100 g.) Serum	Kidney
1, High Ca diet	*Injected ♂		15·1	—
	†Injected ♀		13·0	—
	†Control ♂		13·6	—
2, Moderate Ca diet	Squeaker ♂	Injected	10·0	9·8
	Fanny ♀	Injected	13·4	20·3
	Brown & White ♂	Injected	12·0	332·0
	Tiny ♀	Control	9·8	—
	Patches ♂	Control	11·4	9·1
	Brown & White ♀	Control	11·2	7·1
3, Low Ca diet	White ♂	Injected	7·2	—
	Black ♂	Injected	7·2	—
	Brown ♂	Control	9·6	—
	White ♀	Control	8·5	—

* Age 107 days. † Age 170 days.

Table 4. *Water and fat content of bones of dogs on a moderate calcium intake and treated with parathyroid extract (litter 2)*

	Water content (%) of Femur	Humerus	Tibia
Injected			
Squeaker	60·2	59·5	59·2
Fanny	58·3	58·0	56·0
Brown and White	55·2	55·9	52·2
Control			
Tiny	56·5	57·6	52·1
Patches	50·3	50·3	47·5
Brown and White	49·5	49·6	45·5
	Fat content (%) of		
	Femur	Humerus	Tibia
Injected			
Squeaker	4·7	5·6	4·8
Fanny	1·9	3·0	1·3
Brown and White	3·6	4·4	5·3
Control			
Tiny	7·1	5·8	7·8
Patches	7·9	6·4	7·8
Brown and White	5·5	6·7	5·4

marrow cavities in bones from the injected animals looked larger, but accurate measurement could not be made. The decrease in the ash content of the injected bones which was 2% was not significant. The proportion of dry cancellous bone in the injected animals was increased rather than decreased (cf. Bauer *et al.* 1929) (Table 1, cols. 11, 12). The individual tissues showed no evidence of change in calcification (Table 2). The injections had not therefore changed calcification rate or bone growth.

Bülbring (1931), Selye (1932), Taylor (1935), Burrows (1938) and Silberberg & Silberberg (1943) suggest that parathyroid hormone merely accelerates the mineral turnover in the bones, so that with adequate supply of calcium, bone of normal or high calcium content is built up but is otherwise immature. Burns & Henderson (1936) showed that in newly laid-down bone the proportion of calcium combined with carbonate was less than in fully formed bone. There was no difference in this respect between injected and non-injected animals in litters 2 and 3 (Table 2), but in litter 1 in twelve pairs of estimations on femur, humerus and tibia of injected and control animals, the carbonate combined with calcium was definitely lower in ten, and unchanged in the other two (both of which appear in Table 2). The bones of the injected animal may therefore have been more rapidly resorbed and rebuilt than in the control animal.

Citrate content of bone

Citrate occurs in bone and might be concerned in calcium deposition or removal (Dickens, 1941). A few estimations were made by his method (Table 5). The data are not sufficient for any conclusion to be drawn; they suggest that there is less citrate on the low calcium (and probably low citrate) diet, and a little more in the injected animals on the moderate calcium diet.

Table 5. *Citrate content of dry fat-free femur*

Litter and diet	Treatment	Animal	Citrate content (g./100 g.) of Cortex middle		Cancellous
2, Moderate Ca	Injected	Squeaker	1·21		1·43
		Fanny	1·86		1·56
		Brown and White	1·29	1·66*	1·64
	Control	Tiny	0·98		1·22
		Patches	1·31		1·13
		Brown and White	1·26	1·31*	1·25
3, Low Ca	Injected	White ♂	1·13		1·26
		Black ♂			0·97
	Control	Brown	1·14		1·10
		White ♀	1·14		1·28

* Tibiae, from Dickens (1941).

Serum calcium

The figures given in Table 3 refer to the level at death, some time after the last injection. Several estimations were done on the sera of animals in litters 1 and 2, and the extract soon failed to raise the serum calcium much, though it was still active in a normal animal.

Both older animals in litter 1 had a serum calcium of 13 mg./100 ml. In litter 2, the smallest pair of pups had a serum calcium of about 10 mg./100 ml.: the calcium content of the kidney of the treated

animal was low. The four animals which grew better had a higher serum calcium, the injected ones very slightly higher than the controls. The figures for the kidneys of these two indicated calcium deposits. In litter 3, the serum calcium, while low in all the animals, in the injected ones had fallen to 7·2 mg./100 ml., i.e. to tetany level. They showed no signs of active tetany, but when killed were in a state of extreme depression.

DISCUSSION

Under our conditions the injection of parathyroid extract had no measurable chemical effect on bone growth and calcification, but neither did it produce the softening, bowing, stunting or fracture of bones such as were found by Jaffe & Bodansky (1930). On the other hand, the influence of the injections on the blood calcium shows more similarity. In each case, injections given to animals on low calcium intake brought serum calcium down to tetany level. With higher calcium intakes this fall did not occur. A single dose raised the serum calcium from 11·4 to 15·2 mg./100 ml., but continued injection left the serum calcium little higher in injected than control animals. Jaffe & Bodansky (1930) based their conclusions that the injections brought about decalcification of bone on the gross appearances of the bone and the histological evidence of fibrotic changes in the bone, with increased activity of bone cells. They noted considerable development of new bone in the animals maintained alive for some time. They related the low serum calcium of the treated animals on low calcium intakes to the exhaustion of the available calcium stores of the bone. Our work seems to negative this, since low serum calcium was found when the bone retained ample calcium stores, unless it is assumed that bone calcium is only available to raise the level of serum calcium if it is present in some hitherto unsuspected form. We found no evidence to support the suggestion made by Selye (1932) that the low serum calcium in these animals was due to increased bone apposition, although a drop in serum calcium from 9·0 to 7·2 mg./100 ml. might not be accompanied by a measurable increase in bone calcium. It seems more probable that the parathyroid influences both the building up and breaking down of bone as suggested by the workers previously mentioned, and that the final equilibrium between bone and blood calcium is determined by bone-cell activity and calcium intake.

The dogs used by Jaffe & Bodansky (1930) and Johnson (1932) were about the same age and size as ours. The diet differed mainly in that our dogs had a fairly steady level of calcium intake in each experiment, while theirs varied markedly at different stages of the same experiment. In our experiments, litters 1 and 2 were injected on alternate days. Litter 3, which had a very low calcium intake, was injected daily after the first injections, as these dogs seemed able to tolerate huge doses. Our dogs therefore enjoyed more stable conditions—a fairly constant calcium intake and a steadily increasing dose of the hormone. The other workers' dogs received very varying quantities of calcium and very varying doses of hormone throughout the experiments, and several displayed phenomena of overdosage which necessitated the temporary cessation of the injections. Some of their animals on diets low in calcium showed signs of tetany and were then given large doses of calcium by stomach tube, with further varying doses later. Their animals must therefore have had to adapt themselves during any one experiment to very great changes in calcium metabolism due both to variations in diet and in dose of hormone. Disorganization of bone growth might thus be brought about, but this is not necessarily decalcification. Cox & Imboden (1936) produced soft, thick, knobbed bones, easily liable to fracture, in rats on a high phosphate intake, but these bones had a normal ash content. The variety in bone lesions reported clinically in cases associated with hyperparathyroidism may perhaps be partly explained by fluctuations in the activity of the gland and variations in food calcium.

At a fairly steady level of calcium intake, with a steady increase in the amount of hormone injected, our animals were able to adapt their bone metabolism regularly, and disorganization of bone did not occur. It has been shown that the increased excretion of calcium which follows injection of parathyroid is not maintained except on a diet which itself gives a negative balance (Albright, Bauer, Ropes & Aub, 1929; Greenwald & Gross, 1926). Under regular conditions of diet and injections the hormone merely increases the rate of bone resorption and repair without influencing the chemical composition of the bone laid down.

SUMMARY

1. In puppies, the amount of calcium laid down in the bones and the level of serum calcium varied directly with the calcium intake.

2. The prolonged injection of parathyroid extract did not change the degree of calcification of the bones of puppies on diets rich, adequate or poor in calcium.

3. Injections of parathyroid extract soon ceased to raise blood calcium of puppies on the diets rich or adequate in calcium.

4. A diet of low calcium content caused a decrease in serum calcium and profound depression in the parathyroid-treated puppies.

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The β-Amylase of Barley

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(*Received* 1 *April* 1946)

The amylase of the resting barley grain is known to consist almost entirely of β-amylase, the form which promotes hydrolytic fission of starch into β-maltose and a limit dextrin (α-amylodextrin) (Baker, 1902). During ripening, and again during germination, the α-amylase also is active but its activity is minimal at the resting stage and, in exceptional cases, has been reported to be nil (Hills & Bailey, 1938).

The hypothesis that β-amylases attack the molecules of starch at the non-reducing ends of the glucosidic chains, removing maltose molecules successively, one at a time from each chain or branch, seems to be well established (Ohlsson, 1926; Hanes, 1937). Most starches are now considered to consist of 'amylose', the molecules of which are unbranched, and 'amylopectin' consisting of branched or laminated molecular chains. The action of β-amylase on the former should be complete, 100 % of the theoretical yield of maltose being obtained, whereas its action on the latter is brought to a standstill at the near approach of a branching point (Haworth, Kitchen & Peat, 1944). On these premises we can deduce the proportions of the amylopectin molecule contained respectively in the branchings and other parts provided we know (*a*) the yield of maltose from the pure amylopectin fraction or from the starch by the action of β-amylase and (in the latter case) (*b*) the percentage of amylose in that starch. For this reason alone, the purification of β-amylase and the determination of the exact limit of its action are of importance. Furthermore, with pure β-amylase it would be possible both to define more accurately and to prepare the limit dextrin whose investigation by end-group assay and other methods would be of great value.

The limit of maltose formation from native and soluble starches by β-amylase (usually barley amylases of varying purity) recorded in the literature varies from 59 to 67 % (Hanes, 1937; Blom, Bak & Braae, 1936). By pretreatment of an aqueous extract of barley with acetic acid at pH 3·6 and 0°, Blom *et al.* claimed to have freed the preparation from α-amylase. When such barley extract was allowed to act upon soluble starch at pH 3·3–3·5 at 25° the limit of action was sharply defined at $53{\cdot}1 \pm 0{\cdot}5$ %, and was independent of enzyme concentration and the presence of added maltose. This important observation has encouraged some workers, but by no means all, to adopt similar precautions in preparing β-enzyme from cereal grains. In particular, Kneen, Sandstedt & Hollenbeck (1943) freed β-amylase from α at pH 3·3 at 30° after dialysis. However, the barley extract used by Blom *et al.* contains much reducing matter which shows a marked increase with time at pH 3·4 and 25°—thus introducing an avoidable source of error. In repeating and extending their work in these laboratories, we have encountered several important points with respect to the preparation of the enzyme and the interpretation of the limit of its action. The important question is whether the hydrolysis of starch by pure β-amylase is complete at the 53 or the 60 % stage. If the former, then enzyme preparations which yield 60 % may contain an α-amylase impurity. Alternatively, the 53 %

limit, which was obtained by Blom *et al.* only in acid solutions at pH 3·3–3·5, may be due to some change in the substrate such as a form of retrogradation. The latter phenomenon is known to be encouraged by acid reactions.

A reaction mixture hydrolyzed by β-amylase to its limit possesses reducing power (R.P.) due to the maltose present, the residual dextrin and the enzyme preparation. The latter must obviously be deducted, that of the dextrin is negligible, but there remains a consideration of the R.P. of the original starch. Blom *et al.* employed soluble starch but it is not clear whether its R.P. value was deducted. Many Lintner and other soluble starches possess R.P. equivalent to 2 or 3% maltose, an amount highly significant when considering the difference between a 53 and 60% stage of hydrolysis. In this work deduction has been carried out since much of the R.P. of soluble starches may be due to maltose or simple dextrins the R.P. of which latter, measured by the hypoiodite method, carries the same value as that of the maltose split from them. It is considered that in the circumstances the *increase* in R.P. during the reaction (after allowing for increase in the enzyme preparation alone) is the truest measure of the number of α-glucosidic bonds split.

METHODS

Substrates. Lintner soluble starches (selected commercial preparations) of R.P. varying from 1·7 to 3·3% of theoretical maltose (by hypoiodite) were boiled with water for 5 min. Starch pastes were prepared by stirring at 90–92° for 1 hr., 0·1% NaCl being included in the liquid to assist dispersion.

Reaction mixtures. These contained 2% (w/v) dry weight of starch, buffered with 0·02 N-phosphate-citrate (pH 3·4 or 4·6) with toluene plus nitrobenzene as antiseptic; incubation temp. was 25°. Reducing powers were determined by hypoiodite titration (Willstätter & Schüdel, 1918; modified by Hinton & Macara, 1924), those of initial substrate and of enzyme preparation being deducted. In certain experiments where it was desirable to have approximately equal initial velocities of reaction (Tables 1 and 4) three different quantities of each enzyme preparation were included in separate reaction mixtures and the one exhibiting the most suitable reaction velocity selected for continuation, the others being discarded.

Important precautions. In withdrawing duplicate samples from the reaction mixture the pipette must be free from all traces of iodine which may have contaminated the jet when running the previous withdrawals into the standard iodine. The top of the pipette should contain a cotton-wool plug to prevent possible contamination of the reaction mixture with saliva, a potent source of α-amylase.

Buffers should be freshly prepared. A stock solution of phosphate citrate buffer at pH 3·4 which had been stored for some months developed a small growth of white mould from which enzymes such as α-amylase or maltase might conceivably gain access to reaction mixtures.

Toluene was found to be imperfect as a bacterial antiseptic; nitrobenzene prevented growth of bacteria but not of moulds and yeasts. Toluene and nitrobenzene employed simultaneously were effective. Care must be taken in the hypoiodite titration to withdraw only the quantity of antiseptic dissolved in the water.

Controls for the action of the enzyme on the constituents of its preparation and on maltose were performed under parallel conditions with each experiment and the necessary corrections made. If maltase activity was found, the results of the experiment were rejected. None was detected in the barley mostly used (English of the 1938 season, kiln dried). Occasionally in more recent work with other barley samples a small maltase activity at both pH 3·4 and 4·6 has been detected in prolonged reactions.

RESULTS

The β-amylase preparation of Blom et al. (1936). Ground barley was extracted with water at 50°, the extract purified by freezing and after discarding large quantities of precipitated matter, brought to pH 3·6 with acetic acid and stored at 0°. This preparation hydrolyzed Lintner starch at pH 3·4 to 53% of the theoretically obtainable maltose (R.M. value), whereas the non-acidified extract acting at pH 4·4 yielded 63·0% R.M. The enzyme dose was varied and more than one specimen of barley and of Lintner starch were employed, but the limit at pH 3·4 was always $53{\cdot}0 \pm 0{\cdot}8$%. The enzyme was still active when the limit was reached but would not carry the hydrolysis further even when the pH was changed to 5·0. Thus the experimental work of Blom *et al.* with β-amylase had been repeated and their results fully confirmed.

Further investigation showed that barley extract if stored at pH 3·4 for 24 hr. or more but allowed to act on starch at pH 4·6 would promote hydrolysis beyond 53% and gave variable and indefinite limits between 53 and 60% R.M. It was noted that the R.P. of the barley extract in the reaction mixtures in all the above experiments was equivalent to about 12% of the theoretical maltose equivalent of the starch present. This involved a high 'control'. It was decided to use a purer enzyme preparation and the next investigations were devoted to methods of securing this.

Purification of β-amylase

Freezing aqueous extracts. Alternate freezing and thawing were unsuccessful. After each freezing the ice portion was separated, thawed, refrozen and the ice fraction again separated, this being repeated several times. Similarly the first liquid portion was frozen and the new liquid fraction recovered, refrozen, etc. The final liquid portion was found to be only 1·17 times as active as the final ice fraction, though much precipitated matter had been rejected. There were indications, however, that the final liquid preparation was freer from α-amylase than the original extract.

Extraction of β-amylase from resting barley. The conditions of extraction were investigated and the activities of the extracts (measured by standardized procedure) expressed

as % of theoretically obtainable maltose (R.M.) formed. It was found that both at 25 and 50° the enzyme was rapidly extracted by water in the first few minutes and at decreasing rates with the progress of time. At 25°, extraction was about two-thirds as fast as at 50°; and in both cases the extract promoted hydrolysis to 60% R.M.

100 g. of ground barley were extracted at room temperature with 200 ml. of 20% (v/v) ethanol for 1 hr. (extract A). The solid residue was re-extracted similarly twice (the combined extracts becoming extract B) and again for two further successive 2 hr. periods yielding extract C. Extracts A, B and C were precipitated in 80% (v/v) ethanol and the washed and dried enzyme quantitatively recovered from each. The residue of extracted barley was dried, divided, and the halves extracted respectively with water (D) and 1% papain (E) and similar precipitates from these extracts were prepared. The activities of the five preparations were found to be (relatively): A, 100; B, 26; C, 15; D, 1·3; E, 6; D and E being half portions. D and especially E carried starch hydrolysis further, to 65% R.M. with corresponding loss of iodine coloration. For a yield of enzyme relatively free from α-amylase a short extraction with 20% (v/v) ethanol is indicated.

Precipitation of β-amylase by ethanol. Many precipitants have been used but ethanol possesses certain advantages, notably the power of inactivating the α-form. Aqueous extracts were precipitated fractionally: A, with 50% (v/v) ethanol; B, the filtrate from A raised to 65%; C, the filtrate from B raised to 83% (v/v) ethanol. The activities of the precipitated fractions were as follows (aqueous extract 100): A, 10; B, 50; C, 12; and whilst the hydrolysis limits for the aqueous extract and A were 59–60%, those for B and C were 56% under parallel conditions. It seems that preliminary precipitation by 50% (v/v) ethanol removes relatively little β-amylase, some α-amylase and, incidentally, a quantity of inactive matter. The subsequent raising to 83% gives the purest and most useful fraction. Similar conclusions for barley malt were made by van Klinkenberg (1931) and Caldwell & Doebbeling (1935) and for ungerminated wheat by Meyer, Bernfeld & Press (1940).

Precautions as to sterility. Gelatin plate cultures of washings of barley gave colonies of *B. mesentericus*, and *B. subtilis*, spore-forming thermophilic rods known to secrete α-amylase. The first ethanolic precipitates from barley extracts contain such rods, which even if dead may have excreted amylase into the medium during the aqueous extraction. Ordinary barley carries a microflora beneath the paleae, so that washing before grinding may not remove all bacteria. That such bacteria secrete α-amylase has been experimentally demonstrated in these laboratories. Fortunately, acidification of the extracts or preparations to pH 3·4 inactivates α-bacterial amylases.

Final preparation of β-amylase

As a result of the above experiments, β-amylase was prepared by extracting ground barley with 20% (v/v) ethanol with mechanical stirring for not more than 30 min. at room temperature, centrifuging, filtering and raising the ethanol content to 50%. After settling overnight, the clear portion was treated with ethanol to 80%, the precipitate collected, washed and dried *in vacuo*. An aqueous extract of it was cooled to 0–1°, and simultaneously, sufficient acid to bring it to pH 3·4 and some phosphate citrate buffer at pH 3·4. Then, at 1°, the buffer followed by the acid were added to the enzyme and the mixture kept (with antiseptics) for 24 hr. or more until required for use, when the calculated amount of alkali was added if necessary before allowing it to assume ordinary temperature.

Effect of time of exposure to acid. The effect of exposure to an acid pH of 3·4 (Table 1) at 25° is clearly to destroy α-amylase, but storage for short periods at 0° as employed by Ohlsson (1926) in the preparation of 'saccharogenamylase' from malt is not quite safe. Barley samples and enzyme preparations from them vary in this respect. At pH 3·4 the α-amylase is inactivated rapidly and in this case evidently completely within 24 hr., whereas the β-enzyme partly survived and was further weakened after that time. In other experiments a precipitated enzyme lost about 80% of its saccharifying activity in 2 hr. but little thereafter.

Criterion of purity. The criterion of freedom from α-amylase finally adopted was the hydrolysis limit of a fast reaction at pH 4·6 and is based on evidence furnished in Tables 3 and 4 and discussed later. When this limit is 56% R.M. or less, and further pretreatment of the enzyme at pH 3·4 and 0° does not reduce the limit, the β-amylase is regarded as pure.

Table 1. *Pretreatment of precipitated enzyme at* pH 3·4 *and* 0°

Time of pretreatment of enzyme at pH 3·4 ...	1 min.	1 min.	2 hr.	3 hr.	24 hr.	50 hr.	125 hr.
Vol. of enzyme/100 ml. reaction mixture (ml.)	1	1·6	1·8	2·1	5	10	10
pH of reaction mixture	4·6	3·4	3·4	3·4	4·6	4·6	4·6
Time of reaction (hr.)	Amylase action as percentage of theoretically obtainable maltose						
1	42·6	42·0	41·9	43·9	45·8	45·5	—
24	55·9	51·6	51·8	51·8	52·4	—	52·3
46	58·1	52·5	52·1	51·8	—	—	52·7
94	60·6	53·4	53·0	53·0	—	54·1	—
121	—	—	—	—	54·0	54·2	53·6
145	—	—	—	—	54·3	—	—
192	—	—	—	—	54·5	54·4	—

The limit of reactions promoted at pH 3·4 was established by employing a very wide range of enzyme concentration, the smaller doses of active enzyme being made up to the volume of the highest dose with well-boiled and inactivated enzyme. The results are given in Table 2. After 504 hr. action, the surviving enzyme activity was measured by the addition of fresh starch to the concentration of 2 %. The results of 3 hr. renewed action are expressed on the same basis as the others in the table. In the absence of inactivation these latter results should agree with those in the main table for 3 hr., except that allowance should be made for dilution (7:10) of enzyme on adding fresh starch. However, in col. 1 of Table 2 it will be seen that the surviving enzyme could after dilution to 7/10 perform in 3 hr. what the original enzyme performed in 0·5 hr., i.e. about 24 % of the original had survived. These and the corresponding values for the other columns are included in Table 2. Thus an effective enzyme activity had survived in the two mixtures which had received the most initial enzyme and these had reached 53·5–54 % R.M. Although hydrolysis had virtually ceased in the presence of active enzyme, inspection of the results, especially the bottom six lines of Table 2, leads to the conclusion that 54 % is not necessarily the extreme limit of action of β-amylase on this preparation of Lintner soluble starch at pH 3·4. In the three most dilute enzyme systems, before 54 % hydrolysis could be attained, the enzyme became sufficiently inactivated to bring progress to a standstill without attaining the limit. It seems probable that even the highest enzyme doses were acting so slowly at the end that the limit was not actually reached. The figures in Table 2 also confirm proportionality in the early stages between hydrolysis and the product of enzyme concentration and time. The R.M. of the enzyme control, which was deducted in arriving at the figures in the table, constitutes a serious correction under these conditions, increasing markedly with time.

Table 2. *Limit of action of β-amylase at* pH 3·4

Vol. of enzyme preparation/100 ml. of reaction mixture (ml.) ...	10	5	2·5	0·5	0·1	10 (no starch)
Time of reaction (hr.)	Results as percentage of theoretically obtainable maltose					
0	—	—	—	—	—	1·3
0·25	33·3	—	—	—	—	—
0·5	40·1	32·8	24·4	—	—	—
1·0	44·6	40·4	34·4	12·1	—	—
2·5	—	45·9	41·7	24·1	—	—
3	48·2	46·8	43·7	27·0	8·5	—
4	—	—	44·7	31·2	10·9	—
24	49·3	48·4	47·5	43·0	30·6	2·0
96	51·3	49·9	49·0	46·4	39·4	2·6
144	52·1	51·1	50·1	47·4	40·7	3·7
168	53·9	53·0	—	—	—	—
240	53·9	53·1	50·6	48·5	41·3	6·0
312	53·9	53·2	50·5	48·6	41·5	—
504	54·1	53·4	50·6	48·7	41·8	6·0
Fresh starch added 3 hr. later ...	40·0	31·2	14·6	0·4	0·0	2·0
	Amount of enzyme still active after 504 hr.					
(*a*) as percentage of the amount originally added	24	22	10	1·5	0	
(*b*) as ml. of original preparation (compare top line of table)	2·4	1·1	0·25	0·007	0	

Table 3. *Limit of action of β-amylase at* pH 4·6

Vol. of enzyme preparation/100 ml. of reaction mixture (ml.) ...	1·0	0·5	0·4	0·25
Time of reaction	Results as percentage theoretically obtainable maltose			
1 hr.	38·0	27·6	23·1	16·2
24 hr.	53·3	54·2	53·0	51·9
6 days	54·7	54·8	54·2	53·2
13 days	56·4	—	—	—
20 days	—	56·0	54·2	—
27 days	56·5	56·9	55·8	54·3

The limit of action of the enzyme, pre-restricted at pH 3·4 but promoting hydrolysis at pH 4·6 was now determined using a wide range of enzyme additions (see Table 3). The enzyme had been stored at pH 3·4 at 0° for several hours before use. There was considerable residual enzymic activity in the final reaction mixtures in all cases. The limit is not so clear cut when the reaction is carried out at pH 4·6, a slight 'drift' continuing beyond 54 % R.M. as far as 56–57 % in this case. In other experiments, such as the following one, the final limit was about 56–58 %.

Action of β-amylase on different starches. Many different samples of barley were employed at one time or other in these investigations but little difference was found. It was also considered desirable to extend these experiments to other preparations of soluble starch, and to potato starch paste. One example of such is given.

Potato starch paste and a new Lintner soluble starch (commercial preparation) were used as substrates under conditions identical with those of the previous two experiments except that a fresh barley sample was used as source of amylase. The reaction mixtures at pH 3·4 were split after 46 hr., one part receiving a fresh dose of enzyme equal to

Table 4. *Limits of action of β-amylase on potato and soluble starches*

	Potato starch		Soluble starch	
Substrate concentration in reaction mixture (%) ...	1·25		1·55	
pH	3·4	4·6	3·4	4·6
Vol. of enzyme/100 ml. of mixture (ml.)	20	5	20	5
Time of reaction (hr.)	Results as percentage of theoretically obtainable maltose			
0·5	39·1	39·4	41·3	42·5
1·0	—	43·9	46·4	51·2
17·5	51·2	55·0	52·6	53·4
42	51·4	56·8	53·3	54·2
	pH 3·4 pH 4·6		pH 3·4 pH 4·6	
66	53·5 52·0	57·6	53·3 54·4	54·0
90	53·5 54·1	57·8	53·3 55·5	54·4
144	53·7 56·2	58·2	54·6 57·5	54·7

that previously added, the other being brought to pH 4·6 with a small volume of NaOH. The reactions were now continued. The results are shown in Table 4. Whilst the reaction at pH 3·4 reached the same fairly sharp limit as before, the 'drift' at pH 4·6 is again in evidence. The enzyme clearly survived 42 hr. at pH 3·4. This and other experiments also indicated that a well-dispersed potato starch paste will give the same small range of limits of hydrolysis as Lintner starches at both values of pH and that Lintner starches vary a little among themselves in this respect.

It was invariably noticed that the reaction mixtures whose hydrolysis terminated at 53–57 % remained fairly clear to the end and the iodine coloration blue, whereas those which reached 60 % and presumably contained a trace of α-amylase flocculated and gave a purple iodine colour. Baker (1902) and Meyer *et al.* (1940) have reported similarly, but Hanes (1935) has observed the same flocculation phenomenon with α-malt amylase, acting alone, and it has been repeatedly observed by us. Presumably it is associated with a certain early reaction product of the α-enzyme.

Since the work already described was performed (in 1938–40) a possible explanation of the failure of β-amylase to promote hydrolysis to 60 % at pH 3·4 has presented itself in the phenomena of retrogradation of starch components, particularly amylose. To test this point 2 % Lintner starch was brought to pH 3·4 and divided. One portion was allowed to stand at room temperature at pH 3·4 for 2 days with antiseptics, another after titration back to pH 4·0, others similarly at pH 5 and 7. Finally, all were adjusted to pH 5 and digested under the same conditions as previously with pre-restricted β-amylase. There was no significant difference between the rates or limits of the enzyme actions. Apparently no retrogradation occurred at pH 3·4 to render the starch more resistant to the amylase. It is even less likely to have occurred in our reaction mixtures at 25° which attained the 50 % stage of hydrolysis in a few hours. It seems, therefore, that the true limit of action of β-amylase from barley is about 56 % ± 1 R.M., and varying somewhat with the starch preparation. Higher figures than this obtained from Lintner soluble and potato starch which have received no abnormal pretreatment must be due to α-amylase which, apart from the contribution of the natural barley-grain microflora, is almost invariably present.

Effects of α-amylase impurity. As an example of the effect of a most minute trace of α-amylase the following experiment is illustrative:

A bacterial amylase made from *B. subtilis* and purified by repeated ammonium sulphate precipitation, kindly supplied by Dr Leo Wallerstein, New York, which has been described by Hopkins & Kulka (1942), was dissolved and a solution (0·1 %) prepared such that 1 ml. possessed α-amylolytic activity equal to that of about 1 g. of average barley malt. A hydrolysis of 2 % soluble starch by β-amylase at pH 4·6 of the type reported earlier in the paper—attaining about 40 % hydrolysis in the first hour—proceeded normally. The addition of 0·001 ml. of the α-amylase solution to another 100 ml. of the reaction mixture modified the hydrolysis only in the final stages after 55 % R.P. had been attained, the final limit being 63 %. The mixed enzymes behaved, in fact, like a typical barley amylase, which had not been pretreated at pH 3·4.

Furthermore, by heating unpurified barley amylase solution to 70° for 15 min. as in Ohlsson's method of preparing α-amylase from malt extract, a preparation possessing α-amylase activity was obtained which hydrolyzed starch in the manner characteristic of such enzymes, as indicated both by kinetics of reducing power formation and of iodine coloration.

Also, ethanol-precipitated barley amylase was inactivated by iodine treatment, 10 ml. (equivalent to 30 g. barley) was treated with 0·4 ml. of 0·1 N-I_2 in KI for 2 hr. at room temperature. The saccharifying power was thereby reduced to 1/4000 of the original. There remained, however, a substantial liquefying power, roughly one-fortieth of that of the original enzyme, as measured by the method of Blom & Bak (1938). Thus the liquefying power, another criterion of α-amylase activity, was relatively concentrated by destruction of the β-amylase, and its presence more easily demonstrated.

Finally, the hypothesis was tested that the α-amylase of ungerminated barley is actually and solely due to the presence of bacteria. Ground barley was extracted for 30 min. only, centrifuged, the supernatant liquid filtered, the clear extract passed through a Chamberland filter and preserved sterile. This was used directly as an enzyme instead of precipitating with ethanol. Action on 2 % soluble starch at pH 4·8 and 25°, commenced and continued as far as possible under sterile conditions, gave the following results (Table 5).

Table 5. *Limit of action of sterile barley extract (Chamberland-filtered)*

	Percentage of theoretically obtainable maltose	
Time	5 ml. extract/200 ml. reaction mixture	20 ml. extract/200 ml. reaction mixture
0·5 hr.	39·8	53·9
2 hr.	53·3	—
4 days	56·9	58·2
7 days	58·6	59·1
14 days	60·8	61·6
21 days	60·9	61·8
28 days	60·9	61·8

Apparently the enzyme which is responsible for extending the hydrolysis limit from 56 to 60 % is not removed by these precautions, and it is not solely due to living bacteria. There remained the possibility that amylase-secreting organisms present in the barley may have excreted α-amylase into the medium during the extraction. However, similar results were obtained using commercial pearl barley (i.e. possessing no 'husks' beneath which bacteria could lurk) which was first repeatedly washed with water containing chloroform, rapidly dried in a draught, ground and extracted with water containing antiseptic. This process removed bacteria before the initial ½ hr. extraction. These precautions, whilst advisable, are probably rendered unnecessary by subsequent treatment of the enzyme at pH 3·4.

The conclusion is drawn that all the resting barleys we have examined contain a little active α-amylase. Only barley amylase preparations which have been treated to inactivate the α-enzyme can be employed as pure β-amylase.

SUMMARY

1. The amylase of resting barley grain is mostly β-amylase but in all cases examined contained a trace of α-amylase. Unless adequate precautions are taken, bacteria present on the grain contribute to the α-amylolytic activity.

2. The β-amylase may be prepared free from α by the method of acidification to pH 3·6 applied by Blom *et al.* (1936) to aqueous extracts of barley. This, with precautions that are described, may also be applied to an ethanol-precipitated preparation of the enzyme.

3. Such β-amylase hydrolyzes potato starch paste and Lintner soluble starch alike to 53–54 % of theoretically obtainable maltose at pH 3·4 and to 56–57 % at pH 4·6. The latter is the truer result as considerable enzyme inactivation takes place in the reaction mixtures at pH 3·4. This result is of importance in calculations concerning the amylopectin molecule.

4. These limits vary a little with the starch preparation employed, but retrogradation phenomena are not responsible for them.

5. The many and confusing data in the literature concerning the limit of barley amylase action are largely accounted for by the presence of α-amylase which is responsible for the hydrolysis beyond 56 %. Maltase also may have contributed in some cases.

6. Suitable conditions for the extraction of the β-amylase from the grain and subsequent removal of α-amylase are described.

One of us (R.H.M.) is indebted to the Department of Scientific and Industrial Research for a grant.

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Observations upon the Relation between Sulphydryl Groups and Pyruvate Oxidation in Brain Tissue

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(*Received* 29 *March* 1946)

It is stated in a parallel report (Peters, Sinclair & Thompson, 1946) that work in this laboratory upon the problem of finding antidotes to vesicants was initially based upon two ideas, (*a*) that the pyruvate oxidase system was specially sensitive to arsenicals and (*b*) that the attack was primarily upon an —SH group in this system. The present report deals with the latter aspect. Many years ago Walker (1925, 1928), working under the direction of the senior author, found that diphenylchloroarsine and allyl isothiocyanate rapidly abolished the nitroprusside reaction given by the fixed —SH in skin, trachea and in the muscle powder studied by Hopkins & Dixon (1922). Using cyanide he also concluded from indirect evidence that the disappearance of —SH groups was due to combination rather than to oxidation. About the same time Voegtlin, Dyer & Leonard (1923) advanced the view that arsenic specifically attacked the —SH group of the cell. When it was found that the pyruvate oxidation catalyzed by aneurin in brain brei (Peters, 1936*a*, *b*) was inhibited both by dichlorodiethylsulphone and arsenite, it was natural to suggest (Peters, 1937) that the pyruvate oxidase system might have an essential —SH component which was attacked by the poison. This seemed all the more probable since iodoacetate, shown by Dickens (1933) to combine with —SH groups also inhibited pyruvate oxidation. In these experiments glutathione was used to combine with and inactivate uncombined dichlorodiethyl sulphone. In 1939–40 we obtained experimental support for the idea of an essential —SH group using two accepted methods for this analysis, viz. inactivation by an —S—S compound followed by reactivation by —SH (Hopkins & Morgan, 1938) and treatment with sodium maleate (Morgan & Friedmann, 1938). Though circulated privately in January 1941, publication of these facts has been delayed for war reasons.

EXPERIMENTAL

The brain preparations used were the *brei*, the dispersion and a specially made pyruvate dehydrogenase preparation from brain.

Brain dispersion. Following the method of Banga, Ochoa & Peters (1939*a*), freshly excised brain tissue was ground in an ice-cold mortar; when finely dispersed and rather glutinous, 0·9% KCl (ice-cold) was stirred in gradually, starting with a few drops and grinding between additions. The dispersion (12 ml. for two pigeon brains) was filtered through muslin into a cylinder surrounded by ice, and diluted to the required volume (26 ml.) with 0·9% KCl and sufficient M/2 phosphate buffer solution (pH 7·35) to make the final concentration of phosphate M/10. Before use, the dispersion was poured into an ice-cold 25 ml. beaker, from which successive portions of 2 ml. could be pipetted quickly into the respirometer bottles. It was found advisable to stir thoroughly before taking each fresh 2 ml. sample.

Pyruvate dehydrogenase. The term pyruvate dehydrogenase is used here to describe the factor (or factors) catalyzing methylene blue reduction in presence of pyruvate; the change is presumably an oxidation to acetic acid (Lipmann, 1937; Long, 1938). During preparation all the solutions used are kept ice-cold throughout and made up with glass-distilled water. The pigeon brain tissue (cerebral hemispheres and optic lobes from two pigeons) is finely mashed on an ice-cooled plate, washed three times in a 12 ml. centrifuge tube with two volumes of the usual Ringer-phosphate solution (pH 7·3), then three times with Ringer phosphate solution diluted five-fold with water, and finally twice with six-fold diluted Ringer-phosphate. Between each washing the tissue is separated by centrifuging and upon fresh additions the tissue is thoroughly mashed with a glass rod. The tissue swells in the hypotonic solutions, and the object of the washing is to remove as far as possible traces of hydrogen donators without lowering the osmotic pressure to a point at which the system is inactivated. From the time of placing in the first Ringer-phosphate, the whole preparation takes 2–2½ hr.; rise in temperature soon causes inactivation and even standing in the ice-chest overnight causes a fall of 50% and over in activity, so that preparations must be freshly prepared. The inactivation is not due to removal of a soluble component, because there is no reconstitution upon adding the extracts. So far, no better source has been found than brain tissue, in spite of a search over a range of tissues and animals.

The preparation of pyruvate dehydrogenase was modified somewhat from that described by Long & Peters (1939) and has some advantages: Long (1943) has subsequently described another method of preparing dehydrogenase from brain. As washed in our way, there is some residual reduction without added substrate; in the presence of pyruvate and methylene blue, the time for reduction is 6–10 min. at 38°; the time for reduction in absence of added substrate will be about 4·5 times the 'pyruvate' value. Extensive washing of brain preparations until no

residual substrates are present led to such large losses of activity as to be impracticable. Glass distilled water has been used throughout the work.

The inactivation due to the instability of the brain pyruvate dehydrogenase means that during a long exposure to methylene blue at 38° there can be no straight comparison between a short and long reduction time. In our latest series of experiments we compensated for this fall in activity by adding varying amounts of the dye, so that the reduction time did not much exceed 10 min. Unless otherwise stated, we have then calculated for 0·2 ml. methylene blue. This is not strictly accurate, but does not introduce more than a 20% error. If greater accuracy is required, a correction curve can be worked out. In one case (Table 1) the average of 4 determinations for pyruvate and 2 for succinate (both in duplicate) gave the following ratios, calculated to make reduction for 0·2 ml. 0·0002% methylene blue = 10 min. This means that in this case an estimate for pyruvate using 0·1 ml. gave a 21% longer reduction time than one based upon a 0·2 ml. estimate.

Table 1. *Reduction times of pyruvate dehydrogenase preparation from brain* brei *with varying amounts of methylene blue*

		ml. methylene blue		
		0·2	0·1	0·05
Reduction time (min.) calc. for 0·2 ml. methylene blue	Pyruvate (0·0182 M)	10·0	12·1	13·5
	Succinate (0·082 M)	10·0	12·6	15·0

Method of performing the experiments. Methylene blue (0·05–0·2 ml. of 0·0002%) and 0·1 ml. substrate or water were placed in the hollow stoppers of Thunberg tubes; the body of the tubes contained 0·3 ml. of pyruvate dehydrogenase and sufficient Ringer-phosphate (pH 7·3) to make the total volume 1·5–1·7 ml. The contents were mixed as usual after evacuation and a short period of warming and incubated at 38°. In the experiments with cysteine ester, incubations were done anaerobically; enough NaOH was added to bring the solution of the compound to pH 7·3.

RESULTS

Effect of iodoacetate and iodoacetamide. The sensitivity to iodoacetate previously observed on the total system is also a property of the pyruvate dehydrogenase component (Table 2).

Table 2. *Pyruvate dehydrogenase from brain* brei (pH 7·3) *incubated for* 10 *min. with and without iodoacetate before addition of methylene blue*

Substrate	Reduction times (min.)		
	− Iodoacetate	+ Iodoacetate (1·0 mM)	% change
Pyruvate (0·0182 M)	14	30	−47
Hexosediphosphate (0·021 M)	30	105	−70

At this pH, the sensitivity of pyruvate dehydrogenase is intermediate between that of the very sensitive triosephosphate dehydrogenase (Dixon, 1937) and the less sensitive succinodehydrogenase; on the other hand, it is very sensitive to iodoacetamide.

Effect of sodium maleate. Morgan & Friedmann (1938) have shown that sodium maleate forms stable compounds with —SH groups, and that in comparatively high concentration it inhibits succinodehydrogenase. A preliminary experiment, carried out by Miss Banga in this laboratory in 1939, showed that the pyruvate system was poisoned by maleate more readily than succinate dehydrogenase or triosephosphate dehydrogenase. We have investigated this action to throw light on the —SH problem. Table 3 (Exp. 1920) shows that the succinodehydrogenase in a brain dispersion is practically insensitive to 33 mM-maleate. In the presence of added cytochrome *c*, which slightly improves O_2 uptake, maleate showed slight inhibition of succinodehydrogenase at a concentration (16·5 mM) which completely inhibited the pyruvate oxidase system (Exp. 2153). The cytochrome and carrier system are evidently not affected. The fall in oxygen uptake is also accompanied by decreased utilization of pyruvate. In several experiments the respiration with pyruvate + maleate was less than that with maleate alone. A concentration of 4·1 mM-maleate, just sufficed to inhibit the pyruvate system (Exp. 2154) and this was also true for a dialyzed dispersion (Exp. 2155), showing that the effect was not due to diffusible substances. In further experiments it has been found that the limiting amount of maleate required to produce 50% inhibition in a dispersion was approximately $0{\cdot}115 \pm 0{\cdot}04$ mM or 30–50 μg./ml. In a *brei*, the amount was about 10 times larger, due presumably to difficulties in penetration to active centres. The brain pyruvate dehydrogenase is also sensitive to maleate; 0·04 mM produced 34% decrease in reduction time of methylene blue. The experiments indicate a marked difference in the activity of the —SH groups in succinodehydrogenase and pyruvate dehydrogenase.

We have satisfied ourselves that the action of the maleate is not upon any soluble —SH component by three lines of evidence: (*a*) the maleate (Exp. 2154) also inhibits a dialyzed dispersion; (*b*) glutathione (equivalent to 3 times the maleate concentration) and added after 10 min. does not influence the toxicity developed by 100 μg. of neutralized maleic acid added to the dispersion; (*c*) the compound of cysteine ethyl ester + Na maleate was not toxic at a concentration equivalent to a toxic amount of Na maleate.

Table 3. *Effect of maleate upon oxygen uptake in presence of succinate* (0·067 M) *and pyruvate* (0·011 M) (*brain dispersion*)

Exp.	Maleate concentration (mM)	Rate of O_2 uptake μl./g./hr. (30 min. period) Residual	+Na succinate	Difference with succinate	+Pyruvate	Difference with pyruvate
1920	0	668	2181	+1513	—	—
	16·5	388	1949	+1561	—	—
	33·0	466	1879	+1413 (Change nil)	—	—
2153*	0	922	2897	+1975	2010	+1088
	16·5	691	2257	+1566 (Change −21%)	507	− 184 (Change −100%)
2154*	0	736	—	—	2198	+1462
	4·1	637	—	—	563	− 74 (Change −100%)
2155†	0	182	—	—	1026	+ 844
	4·1	122	—	—	239	+ 117 (Change −86%)

* Cytochrome *c* added.

† Dialyzed 4 hr. at 2° against 0·4% KCl. Fumarate (0·005 M), adenylic acid (0·28 mM), Mg and phosphate added (see Banga *et al.* (1939*b*).

Effects of —S—S *compounds.* Following the experiments of Hopkins & Morgan (1938) upon succinodehydrogenase, pyruvate dehydrogenase has been treated with cystine ethyl ester at 38° and then washed three times with Ringer phosphate. It was then compared with a control similarly treated in all respects except that the cystine ester had been omitted. The latter was used because of its general convenience and the inaccessibility of glutathione. Following the inactivation observed with the —S—S compound, we have found a return of much of the activity by a further treatment with cysteine ethyl ester.

As it was necessary to incubate the pyruvate dehydrogenase for varying periods at 38°, it was essential to correct for changes in activity due to instability alone. Table 4 gives data for this, using varying amounts of methylene blue. Table 5 shows the results of 3 experiments with cystine and

Table 4. *Effect of temperature* (38°) *upon activity of brain pyruvate dehydrogenase*

(Amounts of methylene blue (1/5000) varied to reduce times in bath, pyruvate concentration (0·0182 M) not varied.)

	Methylene blue (ml.)	Reduction time (min.)	Calculated time for 0·2 ml. methylene blue	% activity remaining
No previous incubation	0·2	7	7	100
Incubated for 10 min.	0·2	9	9	78
Incubated for 20 min.	0·1	6¾	13·5	52
Incubated for 30 min.	0·05	11	44	16

Note. Without pyruvate, this preparation originally reduced 0·2 ml. methylene blue in 25 min.

(Some other work was carried out with the sample of tissue used in this experiment. A further experiment with 0·4% KCl lowered the reduction time with pyruvate to 10 min., i.e. it showed a 30% loss of activity. In experiments carried out at 20° instead of 38°, it was found that the time of reduction was increased by approximately 50%; on the other hand, the rate of heat inactivation was reduced; a loss of 30% occurred in 20 min., which was about half that at 38°.)

Table 5. *Effect of treatment with cystine and subsequently with cysteine upon the activity of brain pyruvate dehydrogenase*

(Times for reduction of 0·2 ml. methylene blue (1/5000) at 38°, and for 0·3 ml. tissue sample.)

Exp.	Pyruvate only (min.)	After treatment with cystine (min.)	After further treatment with cysteine (min.)
25	28*	130†	50†
27	2*	74‡	25‡
29	16§	252‡	53‡

* 50% allowed for effect of incubation.

† Incubated for 15 min.

‡ Incubated for 10 min.

§ Control treated aerobically, washed, etc., as in the cystine-treated, but without this addition.

Note. Cystine and cysteine were added as esters, to a final concentration of approximately 0·054 M-cysteine and 0·027 M-cystine. The estimates of reducing times were based upon observation with 0·3 and 0·6 ml. of samples where necessary; as an example, in Exp. 29, the cystine-treated sample gave infinity with 0·3 ml. and 126 min. with 0·6 ml. The actual time calculated to zero activity would be shorter owing to the gradual inactivation in the bath; but there can be no doubt about the 'cysteine-treated' reactivation, which gave 25 min. for 0·6 ml. and 57 min. for 0·3 ml.

cysteine (as ethyl ester); other experiments gave similar results; the inactivation with the —S—S compound and reactivation with —SH was definite.

DISCUSSION

Systematic investigations upon —SH groups in an enzyme appear to have been first made upon urease by Hellerman, Perkins & Clark (1933) (see Hellerman, 1937).

Some facts as to the action of maleate upon brain tissue have already been published by Weil-Malherbe (1938), who quoted earlier literature. He found that 20 mM-maleic acid inhibited the respiration of brain slices in bicarbonate-glucose-Ringer by 10–50%; the inhibition was comparatively small in presence of pyruvate, and it thus appears that the pyruvate oxidase system is even less sensitive in the slice than in the *brei*. Our facts are consistent with the idea that an —SH group is essential for the activity of this system, and that this group is so activated as to be specially sensitive to maleate. It is still necessary to qualify this by pointing out that the evidence is indirect, because the enzyme concerned has not yet been obtained in pure form; the evidence has been much strengthened by the dithiol theory of Stocken & Thompson (1940–41) (for a brief account see Peters, Stocken & Thompson, 1945). (Recently, Barron & Singer (1945), see also Waters & Stock (1945), have also concluded that the pyruvate oxidase system is specially sensitive to —SH reagents; independently Bacq (1942) has found the —SH fraction in proteins abolished by some vesicants.)

SUMMARY

1. Arising from earlier work on chemical warfare agents, and from a research upon antidotes, the sensitivity of the pyruvate oxidase system from brain to some —SH reagents was investigated.

2. The pyruvate oxidase system and the pyruvate dehydrogenase component were much more sensitive to sodium maleate than succinodehydrogenase. Pyruvate dehydrogenase was inactivated by cystine ester and reactivated by cysteine ester. Both these effects are explained by the presence of an essential —SH group in the enzyme concerned.

We are grateful to Dr L. A. Stocken for the preparations of the esters of cystine and cysteine (as hydrochloride).

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An Analysis of the Inhibition of Pyruvate Oxidation by Arsenicals in Relation to the Enzyme Theory of Vesication

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(*Received* 5 *April* 1946)

This research was planned as the first step in an attempt to find an antidote to arsenical vesicants, such as lewisite, and a brief account of the work as a whole has appeared already (Peters, Stocken & Thompson, 1945).

The vesicant action of arsenicals (as of other substances) on human skin will be conditioned by their ability to penetrate the keratin layer of the epidermis, and so reach the site, whether it be small blood vessels or living cell layers, which after

injury give rise to the blister. Thus, although casual contact of the skin with arsenious oxide is not injurious in man, prolonged and intimate contact with this compound may produce erythema and vesication (Cushny, 1924). The arsenical vesicants such as lewisite (chlorovinyldichloroarsine) on the other hand, being lipid-soluble, penetrate the epidermis rapidly, and on reaching the aqueous medium of the cellular layer of the skin will be rapidly hydrolyzed to the corresponding oxides, which are well known to be toxic to Protozoa (McCleland & Peters, 1919; Walker, 1928). These oxides will likewise have general systemic effects if they reach the circulation.

Once the toxic substance has reached the cellular layer of the skin, vesication might theoretically be initiated by several possible mechanisms and may involve selective poisoning of some enzyme as a first step. This hypothesis inspired our work upon this problem, which was based upon two ideas: (*a*) that the pyruvate oxidase system was specially sensitive to arsenicals, and (*b*) that the attack was primarily upon —SH groups in this system. This latter point will be reported separately (Peters & Wakelin, 1946). Evidence for an essential —SH group was early obtained, and eventually made definite by Stocken & Thompson's 'dithiol theory' (1941, 1946). The idea that an attack upon an enzyme may initiate vesication is not new. It certainly occurred to Flury (1921) who thought that the universal cell poisoning by arsenical compounds might be explained by injury to ferments; this was supported by the statement that inhibitory effects of some arsenicals (diphenylchloroarsine) on blood catalase had been found. Rona and colleagues in a series of papers compared the action of several arsenicals upon enzymes; attention was early drawn by them to the possibility of a connexion between the minute amounts of chemical warfare agents causing damage and the small amounts of enzymes; but their work became oriented more to the use of these poisons to throw light upon enzyme constitution. Rona & Szent-Györgyi (1920) found diphenylarsenoxide and phenylarsenoxide very toxic to urease; methylarsenoxide produced about 50% inhibition at 0·01 mM; arsenite was less effective, and atoxyl without action. In contrast, serum lipase was strongly inhibited by atoxyl and arsenite and little affected by methylarsenoxide. Again, Rona, Airila & Lasnitski (1922) found methylarsenoxide to inhibit maltase and α-methylglucosidase *c.* 50% at approximately 0·25 mM, whereas invertase was unaffected. Peters & Walker (1923–5), on the other hand, could obtain no support for the enzyme theory upon the above lines.

Though it has been known for some years, therefore, that trivalent compounds of arsenic are potent inhibitors of some enzyme systems, much of the work fails in essential respects for our problem. For example, solid grounds for believing in an intimate connexion between poisoning of an enzyme and vesication can be based only upon the proof that the action is selective in nature, that the arsenical is active in high dilution, and that some enzyme of more consequence to higher organisms than urease, etc., is inhibited.

Onaka (1911), working in Warburg's laboratory, first linked the poisonous action of arsenic upon whole animals with inhibition of tissue respiration, by showing that 0·029 mM-sodium arsenite inhibited the respiration of nucleated red blood corpuscles. Later, Warburg (1925) suggested that arsenious oxide combines chemically with the iron-containing respiratory ferment, a view not now tenable. Dresel (1926) showed that the rates of respiration of normal rat tissues (liver, kidney and testis) and of Jensen sarcoma were powerfully inhibited by low concentrations of arsenite; both respiration and anaerobic glycolysis of yeast were also sensitive, but the anaerobic glycolysis of Jensen sarcoma was only slightly inhibited. Subsequently (1928), he noted that glucose could protect yeast cells against the inhibitory influence of arsenite on oxygen consumption.

Szent-Györgyi (1930) stated that the oxidation of succinate by minced rabbit liver was relatively insensitive to arsenite, though he found that the respiration of liver pulp was 50% inhibited by 0·13 mM. His general conclusion that 'it had no effect upon oxygen activation and little upon hydrogen activation' was supported by his finding that *p*-phenylenediamine oxidation was insensitive to 10·0 mM-arsenite. In a continuation of this work (Banga, Schneider & Szent-Györgyi, 1931) in which no specific enzyme systems were investigated, they concluded that 'biological oxidations can be classed into two groups, those sensitive to As_2O_3, comprising the greater number, and those unaffected'.

In connexion with Voegtlin's well-known views that the toxic action of arsenic on living cells is due to its reaction with certain essential thiol compounds present in protoplasm, Voegtlin, Rosenthal & Johnson (1931) found that 10 mM-arsenite inhibited the residual respiration of testis by about 25%, and that this inhibition was partially abolished by the addition of excess glutathione. Based only upon the observation that 1·0 mM-arsenite allowed deamination of amino-acids by kidney slices with accumulation of the corresponding α-keto acid, Krebs (1933) claimed that it specifically inhibited α-keto acid oxidation. In 1935, Jowett & Quastel showed that 1·0 mM-arsenite inhibited the formation of acetoacetate from fatty acids by guinea-pig liver, and also decreased the respiration; Quastel & Wheatley (1935) later found that

0·15 mM-arsenite caused 70% inhibition of the breakdown of acetoacetate by kidney slices. Crasnaru & Gavrilescu (1935) found an inhibition of the respiration of brain by novarsenobenzol, in concentrations which, they claimed, stimulated that of other tissues. Arsenite has also been used by Oelkers (1937), Das (1937), Krebs & Johnson (1937), Green & Brosteaux (1936), Ochoa (1941) and Long (1945).

The above review shows that up to the time of our earliest observations in 1936, none of the work upon arsenic and enzymes, except for that of Rona, was upon chemical warfare arsenicals. In general, rather high concentrations of arsenical had been used, and there was little evidence of specificity.

Turning to work in this laboratory, Peters & Thompson (1934) had reported an inhibition with iodoacetate of pyruvate oxidation in brain tissue respiring in the presence of lactate, which was specific for the vitamin B_1-catalyzed system. This was investigated in more detail by Peters, Rydin & Thompson (1935). Later, Peters (1936*a*), drawing attention to the similar vesicant action of iodoacetate and dichlorodiethyl-sulphone, reported that the latter had a similar selective action upon lactate oxidation. The poisoning caused by small concentrations (0·087 mM) gave an accumulation of pyruvate without interfering with the step, lactate → pyruvate; from this it could be inferred that the cytochrome system was untouched. Also in preliminary experiments (1936*b*) Peters found that traces of arsenite exerted a similar effect (0·05 mM producing definite inhibition); using avitaminous brain it was proved that there was genuine inhibition of the pyruvate oxidation. This work was rapidly confirmed and extended in 1939 in experiments described below which place the specific poisoning of the pyruvate oxidase system upon a firm basis.

EXPERIMENTAL

Enzyme preparations. In the aerobic experiments involving enzymes present in the brain, the method has been to compare manometrically the oxygen consumption of pigeon's brain tissue respiring in a Ringer-phosphate solution at pH 7·3 and at 38°, both in the presence and absence of the substrate and of a suitable dilution of the arsenical.

Two methods of preparing the tissue have been used. In the first the cerebrum is finely minced on a porcelain plate. This type of preparation will be referred to hereafter as a 'brain *brei*'. In the second method the whole of the brain except the cerebellum is ground in an ice-cold mortar with the slow addition of 24 ml. ice-cold KCl (0·9%) and 4 ml. phosphate buffer (0·5 M), pH 7·3. It is then pressed through muslin and 2 ml. samples pipetted into the Warburg bottles, which have already been filled with substrate, 0·005 M-Na fumarate and arsenical, and are standing in ice. In the 10 min. interval between death of the bird and immersion of the first bottle in the bath the brain dispersion is maintained at 0–2°. The first reading is taken 20 min. after death, and subsequent readings every 10 min. for the next half-hour. This type of preparation will be referred to as a 'brain dispersion' (Banga, Ochoa & Peters, 1939).

Other enzyme preparations used will be described in a later section.

Arsenicals. Sodium arsenite was prepared by dissolving arsenious oxide (B.D.H., A.R.) in water by warming and addition of N/2 NaOH, subsequently adjusting to pH 7·3.

The lewisite and phenyl- and ethyldichloroarsine (kindly supplied by the Ministry of Supply Experimental Station, Porton) were dissolved directly in Ringer-phosphate; lewisite oxide was dissolved in alkali and neutralized. In each case the dilution was such that the appropriate amount of arsenical was contained in 0·1 ml. of the solution.

RESULTS

Pyruvate oxidation. The result of a typical *brei* experiment with 0·02 M-sodium pyruvate is shown below (Table 1); the inhibitions produced are expressed as percentages of the extra respiration due to added pyruvate. This method of expressing percentage decreases is used throughout the manometric experiments described in this paper.

Table 1. *Effect of* 0·017 mM-*sodium arsenite on pyruvate oxidation in brain* brei

	O_2 uptake (μl. O_2/g. tissue/hr.)			
	0–30 min.	30–60 min.	60–90 min.	
Residual	1025	618	610	
Residual + arsenite	865	592	514	
Pyruvate	2140	1883	1677	
Pyruvate + arsenite	1363	1218	1065	
(Pyruvate-residual)	1115	1265	1067	
(Pyruvate-residual) arsenite	498	626	551	
				Mean
Inhibition (%)	55	50	48	51

The residual respiration, i.e. in the absence of any added substrate, is only slightly affected, whereas the extra respiration due to the added pyruvate is inhibited to the extent of 51% by 0·017 mM-sodium arsenite.

The inhibition produced by arsenite in this system is a rapid process, since the maximum effect is always obtained in the first half-hour of the experiment, and the degree of inhibition remains relatively constant throughout 3 or 4 succeeding half-hour periods. With brain dispersions the inhibition of pyruvate oxidation is almost maximal in the first 10 min. period.

Table 2 shows the percentage inhibition of the oxidation of pyruvate by the brain system in the presence of varying concentrations of sodium arsenite and of three arsenoxides derived from the corresponding dichloro-compounds.

Table 2. *Percentage inhibition of pyruvate oxidation in brain produced by varying concentrations of trivalent arsenicals*

(A) Brain *brei* (mean percentage inhibition over 1½ hr.)

Arsenical	Concentration of As (mM)						
	0·003	0·008	0·017	0·034	0·05	0·068	0·102
Sodium arsenite	—	—	53	75	—	86	95
Chlorovinylarsenoxide	40	60	69	—	76	—	—
Ethylarsenoxide	—	34	—	—	—	—	—
Phenylarsenoxide	—	64	—	—	—	—	—

(B) Brain dispersion (percentage inhibition in first 30 min.)

Arsenical	Concentration of As (mM)									
	0·001	0·002	0·005	0·006	0·008	0·01	0·02	0·04	0·06	0·12
Sodium arsenite	—	—	—	—	—	26	50	88	93	100
Chlorovinylarsenoxide	—	35	100	—	—	100	100	—	—	—
Ethylarsenoxide	—	—	—	65	—	100	100	—	—	—
Phenylarsenoxide	30	58	—	—	98	100	100	—	—	—

The oxides derived from the three arsenical vesicants lewisite and ethyl- and phenyldichloroarsine, are all strongly inhibitory in low concentrations, being even more potent in their effects than sodium arsenite. The dispersion is more sensitive than brei to these compounds.

The inhibition of oxygen uptake is associated with a diminished removal of pyruvate, and is therefore a genuine effect upon pyruvate oxidation. Table 3 gives data for pyruvate, as estimated by bisulphite-binding capacity (Clift & Cook, 1932).

Table 3. *Effect of sodium arsenite upon the aerobic removal of pyruvate by brain dispersions*

	Concentration of As (mM)					
	0·0	0·01	0·02	0·04	0·06	0·12
Pyruvic acid removed (μg./hr.)	470	450	280	150	140	60
Inhibition (%)	0	4	40	68	70	87
Oxygen uptake (μl.)	110	83	58·5	20·5	10	0
Inhibition (%)	0	25	47	81	91	100

Having confirmed the sensitivity of the pyruvate oxidase system to arsenite and to some of the chemical warfare arsenicals, the selectivity of the arsenicals was further defined by investigating their effect on the oxidation of certain other intermediaries in carbohydrate metabolism.

Succinate oxidation. In view of the conflicting results reported in the literature sodium succinate was chosen first. In agreement with the earlier work of Szent-Györgyi (1930), the oxidation of succinate by pigeon brain was found to be relatively insensitive to the presence of arsenic, amounts of arsenite or lewisite that produce an almost maximum inhibition of pyruvate oxidation showing only a small effect on succinate (Table 4). In each experiment additional bottles were set up containing pyruvate to give a direct comparison of the

Table 4. *Inhibition of succinate oxidation by brain systems in the presence of arsenite and lewisite*

(Pyruvate given for comparison.)

Arsenical	Concentration As (mM)	Inhibition (%)	
		Succinate (0·055 M)	Pyruvate (0·02 M)
(A) Brain *brei* (percentage inhibition of extra respiration during 1½ hr.)			
Arsenite	0·034	18	79
	0·034	9	68
	0·068	17	86
	0·102	16	95
	0·34	7	100
Lewisite	0·017	5	89
(B) Brain dispersion (percentage inhibition over 0–30 min.)			
Arsenite	0·04	15	44
	0·04	0	46
	0·04	0	73

Table 5. *Inhibition by arsenicals of α-glycerophosphate oxidation by brain*

Arsenical	Concentration As (mM)	Inhibition (%)	
		α-Glycerophosphate (0·037 M)	Pyruvate (0·02 M)
(A) Brain *brei* (percentage inhibition of extra respiration during 1½ hr.)			
Arsenite	0·034	1	89
	0·034	4	75
	0·034	0	76
Lewisite	0·017	6	—
	0·017	0	—
(B) Brain dispersion (percentage inhibition over 0–30 min.)			
Arsenite	0·04	0	40
	0·04	0	46
	0·04	0	73

relative toxicities. From these experiments it may by inferred that cytochrome oxidase is also relatively insensitive, again confirming Szent-Györgyi's conclusions.

α-Glycerophosphate oxidation. Similar experiments have been carried out using sodium α-glycerophosphate (Johnson, 1936), and it will be seen (Table 5) that the oxidation of this compound by brain is also unaffected by amounts of arsenite or lewisite that produce a very substantial effect on pyruvate oxidation.

Lactate oxidation. It was found that low concentrations of arsenic produced a marked inhibition of oxygen uptake by pigeon brain *brei* in the presence of lactate. If pyruvate accumulates as a result of the presence of the arsenical, this result is not surprising, since Green & Brosteaux (1936) have shown that the immediate product of oxidation of lactate (pyruvate) is itself an inhibitor of the lactate dehydrogenase.

The amount of pyruvate present in the various bottles at the end of the respiration period was therefore estimated by the bisulphite-binding method of Clift & Cook (1932), thus giving a measure of the amount of pyruvate that had accumulated or escaped oxidation throughout the respiration period. The results of these experiments are shown in Table 6.

The considerable accumulation of bisulphite-binding substance found in the presence of the arsenical may be assumed from the earlier work of Peters *et al.* (1935) to consist largely of pyruvate (*c.* 85%), defective oxidation of which would account in large measure for the inhibition of oxygen uptake. The same result occurs with the avitaminous pigeon brain *brei*, where there exists a specific fault in pyruvate oxidation. Table 6 records an experiment in which the rather high concentration of arsenite used inhibits the removal of pyruvate catalyzed by aneurin. It also shows that the catatorulin effect of aneurin is abolished by arsenite.

Confirming and extending the earlier results of Green & Brosteaux (1936) with the lactate dehydrogenase of heart muscle, it appears, therefore, that this system in brain is also not appreciably affected by arsenicals in concentrations that produce an almost maximal inhibition of pyruvate oxidation.

Effect of arsenicals on anaerobic systems. From earlier work in this laboratory it is known that the pyruvate oxidase system of brain is less stable than the brain pyruvate dehydrogenase which is only capable of carrying out simultaneous decarboxylation and oxidation to acetic acid (Lipmann, 1937; Long & Peters, 1939). Since less pyruvate is removed anaerobically than aerobically, we next determined which system was more sensitive to arsenite. Using the Thunberg methylene blue technique the effect of arsenite on the pyruvate dehydrogenase was investigated (Table 7), the enzyme preparation being obtained from pigeon brain by the method described by Long & Peters (1939).

A concentration of arsenite (0·12 mM) which produces almost complete inhibition of the total pyruvate oxidase system only produces 30% inhibition of pyruvate dehydrogenase.

In confirmation of this, the rate of removal of pyruvate by a brain dispersion was measured both aerobically and anaerobically. It was found that in 1 hr. 280 μg. of pyruvate were removed anaerobically and 390 μg. aerobically, as judged by estimations of bisulphite-binding capacity; 0·12 mM-arsenite inhibited the anaerobic removal of pyruvate by about 40% and the aerobic removal by about 90%.

Table 6. *Effect of arsenite and lewisite on the oxidation of lactate by pigeon brain* brei

I. *Normal brain*

(A) Total extra O_2 uptake in 2 hr. (μl. O_2/g. tissue) in presence of 0·026 M-lactate as substrate

Exp.	Arsenical	Concentration As (mM)	Lactate	Lactate + As	Difference	Inhibition (%)
1	Arsenite	0·034	1726	487	1239	72
2		0·034	1748	850	898	51
3	Lewisite	0·017	1847	758	1089	59
4		0·017	1686	420	1266	75
5		0·017	1979	643	1336	68

(B) Extra bisulphite-binding substances (as mg. pyruvic acid/g. tissue) accumulating in 2 hr. in presence of 0·026 M-lactate

Exp.	Lactate	Lactate + As	Extra accumulation due to As
1	1·07	3·25	2·18
2	0·40	2·25	1·86
3	0·44	2·09	1·65
4	0·58	1·97	1·39

II. *Aneurin-deficient brain*

Effect of 0·168 mM-arsenite on lactate oxidation and pyruvate accumulation in avitaminous pigeon brain *brei* during a 2 hr. period of respiration

Lactate	Lactate + As	Inhibition (%)	Lactate + aneurin	Lactate + aneurin + As	Inhibition (%)
			O_2 uptake (μl./g./2 hr.)		
2884	1300	45	4102	1286	32

Extra pyruvate (as mg. pyruvic acid/g. tissue) accumulating in 2 hr. due to arsenite

No added vitamin	With added vitamin
1·25	2·98

Table 7. *Effect of arsenite on the pyruvate dehydrogenase of brain*

		Concentration Na arsenite (mM)							
	Control	0	0·014	0·02	0·04	0·12	0·18	0·32	0·4	0·8
Time taken to reduce MB (min.)	78	23·5	28	30·5	33	33·5	38	39	44·5	80
Inhibition (%)	—	0	8	13	17	18	27	28	39	100

(In calculating percentage inhibition two methods were used to correct for the residual reduction; each gave substantially the same result.)

The pyruvate dehydrogenase preparation from brain was also tested with other substrates, and the effects of both arsenite and lewisite studied. The percentage inhibitions obtained with these substrates are shown in Table 8. Using this system it will be seen that pyruvate dehydrogenase is more sensitive than any of the other dehydrogenases tested, though with lewisite the triosephosphate dehydrogenase is equally sensitive in brain.

Table 8. *Percentage inhibition of the brain dehydrogenase preparation by arsenite and lewisite in the presence of various substrates*

Concentration As (mM)	Pyruvate	Lactate	Succinate	α-Glycerophosphate	Hexosediphosphate
			(A) Arsenite		
0·24	60	10	0	0	20
0·48	100	15	0	27	39
			(B) Lewisite		
0·006	50	6	0	0	58
0·12	100	46	100	100	100

Using a dehydrogenase preparation made from pigeon breast muscle it was found that 0·24 mM-arsenite produced no inhibition with succinate, α-glycerophosphate, lactate, fumarate, malate or hexosediphosphate, while an acetone-dried rabbit muscle preparation was not inhibited by 0·8 mM-arsenite in the presence of succinate, glucose, hexosediphosphate, malate or fumarate. This relatively enormous concentration also did not affect the triosephosphate dehydrogenase of pig heart muscle, and 1·2 mM produced no inhibition of pig-kidney amino-acid oxidase (using alanine as substrate).

On account of the importance of the triosephosphate dehydrogenase system, two experiments with this enzyme are given in detail (Table 9). The source of the enzyme was an acetone-dried rabbit muscle powder prepared in collaboration with Dr L. A. Stocken. The activity of the preparation was determined by estimating the CO_2 produced from bicarbonate solution resulting from the catalyzed coenzyme-linked reaction occurring between triosephosphate and pyruvate (Green, Needham & Dixon, 1937). Sodium hexosediphosphate, freshly prepared from the Ca salt by Dr Stocken, was added as a source of triosephosphate which is formed from hexosediphosphate by the zymohexase present in the muscle extract (Meyerhof & Lohmann, 1934).

Using this unpurified preparation, relatively large amounts of lewisite (20 times the amount required to produce a marked effect on the unpurified pyruvate oxidase system) cause no inhibition of triosephosphate dehydrogenase. With pigeon breast muscle preparation, 0·006 mM-lewisite gave no inhibition of lactate, succinate, α-glycerophosphate or triosephosphate dehydrogenases, and actually stimulated the malate dehydrogenase, as was also found by Green (1936) using 30 mM-arsenite.

Lewisite oxide (0·48 mM) had no effect on fumarate, and caused only 20% inhibition of succinate, 0·95 mM producing 35% inhibition of the latter. In general, these other dehydrogenases were very resistant to the arsenicals tried, though succinodehydrogenase was poisoned by high concentrations.

We have seen that the complete pyruvate oxidase system is particularly sensitive to the action of arsenite, and the dehydrogenase, or anaerobic phase, less so. It was probable, therefore, that yeast

Table 9. *Effect of lewisite on triosephosphate dehydrogenase*

(1 ml. enzyme (5% muscle powder), 0·5 ml. 0·3% cozymase, 0·3 ml. 0·05M-hexosediphosphate and 0·1 ml. M-pyruvate. Hexosediphosphate (HDP) tipped in from side-bulbs after temperature equilibration.)

	Concentration As (mM)	μl. CO_2 evolved in 20 min. Exp. 1	Exp. 2
Enz. + cozymase + pyruvate	—	23	42
" " " + HDP	—	148	110
" " " " + lewisite	0·03	161	—
" " " " "	0·3	—	177

carboxylase would also be less sensitive to arsenite since there is no oxidative phase. Experiments were therefore carried out anaerobically at 28° using alkaline-washed yeast as the apo-enzyme and excess vitamin B_1, following the technique of Ochoa & Peters (1938). Cocarboxylase was added as coenzyme. It was found that a concentration of arsenite (0·12 mM) that completely inhibited the oxidation of pyruvate by pigeon brain had no effect at all on yeast carboxylase, and the same result was obtained with 0·02 mM-lewisite and ethylarsenoxide.

Relation of arsenicals to coenzymes. Banga *et al.* (1939) proved that in addition to cocarboxylase, an adenine nucleotide and cozymase were necessary for the pyruvate oxidase system. Table 10 shows that, using the brain dispersion, an excess of cocarboxylase, up to 100 times the necessary amount, has no restorative action on the inhibition.

Table 10. *Effect of excess cocarboxylase upon the inhibition of pyruvate oxidase by arsenite or lewisite*

	Concentration arsenical (mM)	Concentration cocarboxylase (μg./2·5 ml.)	Percentage inhibition of pyruvate oxidation: Without cocarboxylase		Percentage inhibition of pyruvate oxidation: With cocarboxylase	
Arsenite	0·17	40	100		100	
	0·02	40	86	Mean = 60%	86	Mean = 54%
	0·02	50	46		28	
	0·02	50	55		52	
	0·02	18	63		42	
	0·02	—	50		64	
Lewisite	0·014	4	100		100	
	0·010	50	100		100	
	0·001	18	50		47	

It was shown in Table 6 that aneurin does not restore the inhibition. Further, Table 11 shows that adenylic acid is unaffected and that the part of the oxidase system catalyzed by an adenine nucleotide is sensitive to lewisite oxide. Since it is already known that the malate and lactate dehydrogenases for which cozymase is necessary are insensitive to arsenic, it can be concluded that the point of attack lies in the protein part of the enzyme.

Table 11. *Effect of adenylic acid upon the rate of pyruvate respiration in pigeon brain dispersion dialyzed for 2 hr. at 2°*

(Pyruvate, 11 mM; lewisite, 0·04 mM; adenylic acid, 0·28 mM)

	O_2 uptake μl./g./hr.		
Pyruvate	Pyruvate + adenylic acid	Pyruvate + lewisite	Pyruvate + adenylic acid + lewisite
699	2212	177	197

The blood pyruvate level in arsenic intoxication. In the failure of pyruvate oxidation that occurs in animals fed on a vitamin B_1-deficient diet it has been shown (Thompson & Johnson, 1935) that in the terminal stages of the deficiency, when the animals are showing signs of opisthotonus, there is a marked elevation in the bisulphite-binding capacity of the blood due to an accumulation of pyruvate. To determine whether the effect of arsenic on the pyruvate oxidase system is also an *in vivo* action leading to a demonstrable metabolic disorder in the whole animal, the bisulphite-binding capacity and pyruvate level of normal animals have been compared with those of animals at varying stages after the administration of arsenic.

The animals were beheaded and the blood taken directly into 25% (w/v) trichloroacetic acid. Total bisulphite-binding substances were then estimated in the protein-free filtrates by the method of Clift & Cook (1932), and in some experiments the pyruvate level determined by the method of Lu (1939). In the experiments with pigeons, the arsenic was administered intramuscularly in the form of a solution of sodium arsenite (pH 7·3). In the 'acute' experiments the birds received one dose of 8 mg. As_2O_3/kg.; in the 'chronic' experiments the daily dosage was varied according to the condition of the birds.

The results of these blood estimations are given in Table 12. Both the bisulphite-binding capacity and the blood pyruvate level itself are raised by poisoning with arsenite, this increase showing itself

Table 12. *Effect of injected arsenite on the blood-pyruvate level of pigeons*

Normal	Acute	Chronic	Total dose (mg. As_2O_3)
(A) Bisulphite-binding capacity (as mg. pyruvic acid/100 g. blood)			
	(Duration of intoxication given in brackets.)		
4·17	7·32 (2 hr.)	20·52 (13 days)	35·5
3·76	8·85 (2 hr.)	9·60 (15 days)	35
3·90	8·33 (3 hr.)	24·98 (1 day)	8
4·03	10·32 (3 hr.)	21·29 (2 days)	10
3·33		21·87 (2 days)	11
3·36		18·66 (4 days)	8·5
5·24		14·99 (4 days)	8·5
5·26		17·10 (43 days)	7
4·06			
Mean 4·01	8·81	18·63	
(B) Pyruvate, determined by 2:4-dinitrophenylhydrazone method			
	(mg. pyruvic acid/100 g. blood)		
1·28	4·02 (2 hr.)	12·39 (4 days)	8·5
0·94	3·00 (2 hr.)	14·15 (4 days)	8·5
0·94	3·12 (2 hr.)	13·85 (43 days)	7
	6·30 (3 hr.)		
Mean 1·06	4·11	13·47	

as early as 2 hr. after injection of the arsenic. Earlier workers (Johnson & Edwards, 1937; Lu & Platt, 1939) have shown that exercise raises the blood pyruvate level of normal animals, but as the poisoned birds invariably remained resting in their cages, usually in the sitting position, for at least 1 hr. before killing, exercise can safely be excluded as a contributory cause of the findings.

That a similar state of affairs exists in mice and rats after poisoning with arsenite is shown in Table 13.

Table 13. *Blood pyruvate (mg./100 g. blood) of mice and rats poisoned with a single intraperitoneal injection of* As_2O_3

	Mice				Rats	
	Blood pyruvate (mg./100 g.) for As_2O_3 dosage (in μg./g.) of				Blood pyruvate (mg./100 g.) for As_2O_3 dosage (in μg./g.) of	
	0	8	12	15	0	7
Duration of poisoning (hr.)	1·23	—	—	—	1·18	—
	1·23	—	—	—	—	—
	1·38	—	—	—	—	—
1	—	—	—	—	—	2·44*
1½	—	—	—	8·1	—	—
1¾	—	—	2·88	—	—	—
2	—	—	2·32	—	—	1·21
2	—	—	6·00	—	—	—
3½	—	2·12	—	—	—	2·28
4½	—	2·16	—	—	—	—
5	—	1·66	—	—	—	—
5	—	2·33	—	—	—	—
5	—	2·70	—	—	—	—
5	—	3·00	—	—	—	—
7	—	4·45	—	—	—	—
11	—	—	—	—	—	1·75
22½	—	6·55	—	—	—	3·37

* This animal received 10% (w/v) lewisite in ethanol on the shaved skin instead of the As_2O_3.

It will be noted that one experiment is included in Table 13 in which the rat was contaminated with lewisite. This result was confirmed by carrying out bisulphite-binding titrations (Table 14) on the blood of three normal rats and two that had received $4 \times LD_{50}$ of lewisite on the clipped skin of the back.

Table 14. *Bisulphite-binding capacity of blood of lewisite-poisoned rats*

(Duration of intoxication given in brackets.)

Rat no.	Pyruvic acid (mg./100 g. blood)	
	Normal	Poisoned
1	4·52	—
2	6·61	—
3	5·84	—
4	—	7·40 (½ hr.)
5	—	12·93 (1 hr.)

DISCUSSION

The accumulated evidence shows that, among the enzyme systems studied, the pyruvate oxidase system is specially sensitive to poisoning by arsenicals. Though isolated enzymes were studied, the emphasis in this work is laid upon results with unpurified enzyme systems approximating more to whole cell preparations. In this way it was thought that the differential affinity of the different possible 'arsenic acceptors' within the cells could be better determined.

That inactivation of the pyruvate oxidase system is one of the outstanding biochemical effects brought about *in vivo* by arsenicals is supported by two lines of reasoning:

(1) the increase in the blood pyruvate level in poisoned animals;

(2) the similarity between the clinical manifestations of arsenical neuritis and the neuritis accompanying vitamin B_1 deficiency (Sinclair, 1939).

On present evidence the pyruvate oxidation system is found to be outstandingly sensitive, both *in vivo* and *in vitro*, among the enzymes tested with low concentrations of arsenite or lewisite. Hence, we postulate that inactivation of this system is responsible for the primary effect in the lewisite lesion; with higher concentrations there is evidence that other enzymes are attacked, but from the work reported here it was logical to use the pyruvate oxidase system as a test system for arsenical poisoning, a view which subsequent work thoroughly substantiated.

With regard to the application of these results to the original problem of vesication, they provide grounds for the first time for a belief in inactivation of an enzyme as a first step in cell damage, and so support the 'enzyme theory' as one mode at least of inflicting damage in skin. The application of these results to skin itself will be discussed elsewhere (Thompson, 1946). The increased capillary permeability which is the result of damage by lewisite (Cameron, Short & Calder, 1941), may be due either to direct action upon some cell surface, to liberation of capillary-dilating substances from skin cells, or to interference with the metabolism of the capillary endothelial cells; both of these two latter effects could be initiated by enzyme damage; the actual formation of the vesicle clearly involves other stages.

Our results on the pyruvate system have been substantiated and considerably extended by parallel work carried out in U.S.A. by Barron and his colleague (cf. Barron & Singer, 1945). In addition to unreported observations (R. A. P.) much of the work described above was contained in reports submitted earlier to the Ministry of Supply (Sinclair & Thompson, 1940; Sinclair, 1940).

SUMMARY

1. The sensitivity to trivalent arsenicals of several respiratory enzyme systems related to carbohydrate metabolism has been investigated in relation to the 'enzyme theory' of vesication.

2. Of the enzymes studied the pyruvate oxidase system has proved the most sensitive.

3. The pyruvate dehydrogenase is more sensitive than the other dehydrogenases tested, but less so than the whole oxidase system of brain.

4. Lewisite has in general proved more toxic than arsenite.

5. None of the coenzymes at present known to be concerned with the pyruvate oxidase system is sensitive; we therefore conclude that the point of attack is on a protein component of the system.

6. Finally, *in vivo*, the blood pyruvate level is significantly raised in animals poisoned with arsenite or lewisite.

7. It is concluded that solid grounds have been obtained for the belief in an enzyme theory of vesication, and that the first point of attack of arsenicals is on pyruvate oxidation.

This work was initiated at the outbreak of the war for the Ministry of Supply. Our thanks are due to the Chief Scientific Officer, Ministry of Supply, for permission to publish. We are also grateful to Messrs R. W. Wakelin, L. C. Waters, and C. Dear for technical assistance.

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The Effect of Arsenical Vesicants on the Respiration of Skin

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(*Received 5 April* 1946)

It has been known for many years that trivalent compounds of arsenic are potent inhibitors of certain enzyme systems, including some of those involved in cell respiration. Among the arsenic-sensitive enzymes are those responsible for the oxidation of α-keto-acids (Krebs, 1933). It is now known that compounds of arsenic are highly selective in their action in low concentrations on cell enzymes, and it is believed that the pyruvate oxidase enzyme system is prominent among those affected (Peters, 1936; Sinclair & Thompson, 1940); the evidence for this is based both on the high sensitivity of the pyruvate oxidase system of brain to trivalent arsenicals *in vitro*, and on the *in vivo* finding of a rapid rise in the level of the blood pyruvate in animals poisoned with arsenic, analogous to that occurring in vitamin B_1 deficiency (Thompson & Johnson, 1935).

One possible mechanism for initiating skin damage by the arsenical vesicants, leading ultimately to the formation of a vesicle, would be the production by the arsenical of some metabolic disturbance in the skin, such as the blocking of pyruvate oxidation due to the vesicant combining with or in some way inactivating the enzyme concerned. The background of this 'enzyme theory' of vesication, and the facts on which it is based are discussed in an earlier paper (Peters, Sinclair & Thompson, 1946).

It was important therefore to determine whether arsenical vesicants exert the same inhibitory action on the respiration of skin as on that of brain tissue, and whether a correlation exists between the development of visible pathological changes in the skin (erythema or oedema) and any specific inhibitions of oxidations in the skin. Following on the earlier work with brain, a comparison has therefore been made of the respiration of normal skin and of skin to which lewisite had been applied during life, and also of the specific oxidations of pyruvate and succinate in skin under these conditions.

Owing to the relatively large amount of inert tissue in mammalian skin (keratin and elastic fibrous tissue), the respiratory activity of whole skin is low. Thus, Wohlgemuth & Klopstock (1926) found a mean Q_{O_2} (μl. O_2/hr./mg. dry wt.) of 2·1 for human skin obtained from amputated limbs, and for normal skin obtained from plastic operations; a mean value of 1·48 was reported by Amersbach, Nutini & Cook (1941) for human skin taken from below the axilla in the mid-axillary line. Buhmann (1936) obtained an even lower mean value of 1·38 (ranging from 0·5 to 2·5) for normal human skin. Adams (1936), working with adult rat skin, found values ranging from 0·56 to 2·5, while Cook, Kreke & Nutini (1938) obtained values varying from 0·5 to 1·15.

The age of the animals has a marked effect on the respiratory activity of the skin, due probably to variations in the thickness of the keratin layer; thus, Loebel (1925) found for the skin of newly born white mice a Q_{O_2} as high as 6·9, and for the skin of one newly born rat a Q_{O_2} of 3·5. Needham & Dixon (1941), using 2–5-day-old rats, obtained values of the order of 4–5, while Adams (1937) showed that a steady fall in respiratory rate occurred from 10 to 210 days.

The effect of inhibitors on the respiration of skin appears to have received little attention. Huf (1936, 1938), however, has shown that 0·001 M-KCN brings about a substantial decrease in the oxygen uptake of frog's skin. Francis & Gatty (1938) have studied the effect of iodoacetate on the respiration of frog's skin, and have attempted to correlate the inhibition of oxygen uptake with the decrease also brought about by iodoacetate in the electrical p.d. across the skin. It is interesting to note that these workers also observed that arsenite brings about a decrease in the electrical p.d. of skin; arsenite was only used in a few experiments, however, and no details are given.

EXPERIMENTAL

Estimation of enzymic activity. Oxygen consumption was determined manometrically, the skin being sliced and suspended in a Ringer-phosphate solution (containing calcium) at pH 7·3 and at 38°. Owing to the low rate of respiration of skin, between 200 and 300 mg. of skin slices were added to each bottle, and the oxygen uptake measured in duplicate over two successive 1 hr. periods. The initial difficulty experienced in drying the skin slices down to constant weight necessitates the expression of results as μl. O_2/g. tissue (wet wt.)/hr., the mean value throughout the 2 hr. periods of the experiments being given.

Substrates and inhibitors. Two substrates, sodium pyruvate (kindly supplied by Prof. R. A. Peters) and sodium succinate, were used, the final concentrations in the bottles being 0·02 and 0·06 M respectively.

Sodium arsenite and lewisite (kindly supplied by the Ministry of Supply Experimental Station, Porton) were used as inhibitors, and, when added direct to the bottles, the final concentrations were 10^{-4} and 3×10^{-5}M respectively. The sodium arsenite was prepared by dissolving arsenious oxide (British Drug Houses Ltd., A.R.) in water by warming and with the addition of N-NaOH, subsequently adjusting the pH to 7·3. The lewisite was dissolved directly in Ringer-phosphate.

Source of tissue. Rat skin has been used throughout this work. In the earlier experiments adult rats were used; in later experiments young rats (3–4 weeks old) were selected in order to obtain a larger oxygen uptake *in vitro*, while in a few experiments (see Table 2), following the work of Needham & Dixon (1941), baby rats (2–3 days old) were used.

The skin slices were obtained as follows: The rats were first lightly anaesthetized with ether, and a large area of skin on the belly and flanks clipped free from fur. The clipped area was then washed with ethanol and water. Two hours after the end of the anaesthetic, or longer, the animal was killed by decapitation, the skin removed, scraped free from subcutaneous tissue and sliced with a razor.

RESULTS

Respiration of normal skin in the presence of arsenite or lewisite

The effects of the addition of sodium arsenite on the 'residual' respiration (i.e. in the absence of added substrate) of normal skin, and on respiration in the presence of added pyruvate were first studied. Since it has been shown by Sinclair & Thompson (1940) that the succinoxidase system of brain is relatively unaffected by concentrations of arsenite that cause an almost complete inhibition of pyruvate oxidation by brain, succinate has also been used as a substrate in order to determine whether arsenite in low concentrations also exerts a selective action on the respiratory enzymes of skin, or whether there is in this tissue a more generalized inactivation of cellular function.

The values obtained for the oxygen uptake of skin slices respiring in Ringer-phosphate solution agree well with those of earlier workers. The addition of 0·02M-pyruvate to the medium results in a slight but definite increase in oxygen uptake. Succinate is oxidized more rapidly.

In Table 1 may be seen a series of values for the *extra* oxygen uptake brought about by the addition of these substrates to *adult* rat skin slices. One experiment is purposely included in Table 1 in which added pyruvate caused no extra oxygen uptake; a further example is given in Table 3. Such experiments have very occasionally occurred throughout the work on adult skin.

It might be argued that the small and variable extra uptake produced by added pyruvate is not due to oxidation of the pyruvate but to a catalytic effect produced by the pyruvate on some other oxidation system. Sellei & Spiera (1938), however, have shown that the skin normally stores carbohydrate, so that this store may be adequate to maintain the respiration at an almost maximum rate in the absence of any added carbohydrate substrate; it should be noted in this connexion that glucose also brings about only a small extra uptake. Moreover, in view of the central role that pyruvate is now believed to play in carbohydrate metabolism, it is thought likely on present evidence that true oxidation of pyruvate by skin does occur. Peters & Wakelin (1943) brought forward further evidence for this by demonstrating the presence of co-carboxylase in rat skin.

Table 1. *Extra respiration of normal adult rat skin slices due to the presence of added substrates*

	(μl. O_2/g./hr.)	
	Pyruvate	Succinate
	28	104
	20	91
	36	164
	95	137
	45	224
	12	78
	0	123
	49	87
Mean	36	126

The effects produced by arsenite and lewisite on these oxidations are shown in Table 2. In these experiments the arsenical was added to the Ringer-phosphate solution in the bottles (with or without extra substrate), the skin slices added next and their weight determined by re-weighing the bottles. It will be seen that in every case the addition of the arsenical caused an inhibition of the residual respiration and also of the total respiration in the presence of added substrate. When calculated for the extra oxygen consumption due to the added substrate, however, it will be seen that while in every experiment but one pyruvate oxidation has been markedly inhibited (86–100%), succinate oxidation has been only slightly affected (0–19%).

In most cases the respiration was well sustained and the inhibition produced by the arsenical was approximately constant over the two successive periods of 1 hr.

The respiration of lewisite-contaminated skin

It was next of interest to determine whether similar biochemical derangements were present in skin to which an arsenical vesicant had been applied during life.

The procedure adopted was as follows: Male piebald rats, 4–8 months old, were selected. Two large areas of skin (roughly 30 sq.cm.) on either side of each animal were clipped and shaved while

Table 2. *Effect of added arsenicals on pyruvate and succinate oxidation by normal rat skin slices*

(Results as μl. O_2/g./hr.)

Pyruvate

				Inhibition (%)		Extra O_2 uptake in presence of substrate		
R	*RA*	*P*	*PA*	*R*	*P*	(*P*-*R*)	(*PA*-*RA*)	Inhibition (%)
			Arsenite. Adult rats					
112	75	143	76	33	47	31	1	97
124	67	158	58	56	63	34	0	100
200	137	213	138	32	35	13	1	92
170	98	200	143	42	29	30	45	0
129	98	159	91	24	43	30	0	100
148	73	169	76	51	55	21	3	86
141	72	161	66	49	59	20	0	100
149	128	176	112	14	36	27	0	100
172	131	215	114	24	47	43	0	100
135	112	185	115	17	38	50	3	94
			Lewisite. Baby rats					
—	—	399	205	—	49	—		—
—	—	408	171	—	58	—	—	—

Succinate

				Inhibition (%)				
R	*RA*	*S*	*SA*	*R*	*S*	(*S*-*R*)	(*SA*-*RA*)	Inhibition (%)
			Arsenite. Adult rats					
148	73	291	205	51	29	143	132	8
141	72	303	206	49	32	162	134	17
149	128	349	289	14	17	200	161	19
168	139	303	262	17	14	135	123	9
148	74	237	206	50	20	109	132	0

R = residual. *PA* = pyruvate + arsenical.
RA = residual + arsenical. *S* = succinate.
P = pyruvate. *SA* = succinate + arsenical.

the animal was under light ether anaesthesia. Ethanol (0·2 ml.) was dropped from a pipette on to the shaved area of one side, and was spread over the area with a glass rod; 0·2 ml. of a suitable dilution of lewisite in ethanol was then spread over the shaved area on the opposite side. At varying intervals after these applications the animals were killed by decapitation, the skin removed, separated from subcutaneous tissue, and sliced. The oxygen uptake of the slices from the normal side was then compared with that of the slices from the side painted with lewisite; by this means the respiration of the contaminated skin was always compared with that of normal skin from the same animal, thus obviating the extensive differences in respiratory rates that exist among the skins of different animals. Pyruvate and succinate were again used as substrates.

Preliminary experiments were first carried out to determine approximately the lowest concentration of lewisite which, when applied to the shaved rat's skin, would produce visible pathological changes; it was found that 0·2 ml. of 0·1% solution of lewisite in ethanol, when spread over the shaved area produced a visible degree of oedema within 30 min. In the rat, under the conditions of these experiments, oedema of the skin and subcutaneous tissues was the first sign of damage; erythema did not usually occur until later, and was then only faint. Vesication was never seen.

The results of these experiments are summarized in Table 3. In column 1 of Table 3 an approximate estimate of the amount of lewisite applied to unit area of skin is given, calculated from the total weight of skin used. Column 2 shows the time that elapsed between the application of the lewisite and the killing of the animal. It will be seen that if the animal is killed 5 min. after the application of these concentrations of lewisite to the skin there is already a marked inhibition of pyruvate oxidation but no apparent inhibition of succinate oxidation (when calculated as extra O_2 uptake due to the added succinate). With these concentrations of lewisite there are no visible changes in the skin at the end of 5 min. It must be pointed out, however, that although the skin was removed from the

Table 3. *Comparison of the oxidation of pyruvate and succinate by normal skin and by skin contaminated with lewisite*

(Results as μl. O_2/g./hr.)

Approx. amount lewisite (μg./g. skin)	Duration of action (min.)	Residual		Pyruvate		Succinate	
		Normal	Lewisite	Normal	Lewisite	Normal	Lewisite
45	40	190	135	212	148	278	—
60	50	159	87	209	113	388	289
70	5	176	99	217	90	272	264
90	45	195	100	256	134	327	269
95	30	264	136	286	95	346	301
125	25	213	71	246	70	311	178
140	30	235	45	229	44	369	158
150	5	141	87	154	87	235	226
150	5	144	97	172	104	258	218
160	35	127	50	157	42	237	134

Extra respiration in above experiments (i.e. substrate-residual)

Pyruvate		Succinate		Inhibition (%)	
Normal	Lewisite	Normal	Lewisite	Pyruvate	Succinate
22	13	88	—	41	—
50	26	229	201	47	13
41	0	96	165	100	0
61	34	132	169	44	0
22	0	82	165	100	0
33	0	98	107	100	0
0	0	134	113	—	15
13	0	94	139	100	0
28	7	114	121	75	0
30	0	110	84	100	26

animals 5 min. after contamination actual measurements of oxygen consumption were not begun until a further 30–40 min. had elapsed.

In those experiments in which a longer time was allowed to elapse before killing the animal, visible oedema was present in each case. In these experiments also there was a large inhibition of pyruvate oxidation in the lewisite-contaminated skin, whereas succinate oxidation again was only very slightly inhibited or, in some cases, actually stimulated.

If the oedema were responsible for an apparent inhibition of pyruvate oxidation owing to 'dilution' of the enzyme systems, it would be expected that succinate oxidation would be inhibited to the same degree. Although this was not found to be so, in two experiments portions of skin from the normal side and from the side painted with lewisite were dried at 110°; in these two cases the lewisite-contaminated skin contained only 8 and 2 % more water respectively. Moreover, when the skin is removed from the body it is apparent that most of the fluid is present in the subcutaneous tissue. The oedema may therefore be safely excluded as contributing to this diminished respiratory rate by any dilution effect. Concentrations of lewisite of 1 % and higher were found to inhibit both pyruvate and succinate oxidation.

DISCUSSION

The experiments described above indicate that a concentration of lewisite sufficient to produce very considerable oedema when applied to the skin of rats regularly produces a marked fall in the rate of respiration of the contaminated skin. An attempt has been made to analyze this inhibition of respiration in terms of specific oxidations. The interpretation of this aspect of the work is rendered difficult on account of the small and very variable oxygen uptake induced by the presence of added pyruvate. Using 2–5-day-old rat skin Needham & Dixon (1941) have indeed stated that the addition of either glucose or pyruvate failed to cause any effect on oxygen uptake. In the present work, in a very few experiments, as already pointed out, no extra respiration was observed, but in every case where the addition of pyruvate did result in increased respiration, there has been a substantial decrease, amounting to 40–100 %, in the extra uptake of the contaminated skin. This is in striking contrast to the extra respiration induced by the presence of succinate, the oxidation of which is only very slightly inhibited by these concentrations, and is in fact more frequently apparently stimulated. This finding with succinate indicated, therefore, that the damaging action of lewisite on skin is not due to a

generalized denaturant or coagulant effect on the skin proteins. Although the evidence presented above is insufficient to warrant a firm conclusion that vesication is initiated by this respiratory defect, yet, since pyruvate oxidation is known to be a fundamental stage in the energy-yielding reactions of many animal cells, it is felt that inactivation of carbohydrate metabolism at this stage may represent a biochemical lesion sufficiently severe to account for grave alterations in the functions of the capillary endothelial cells. It should be pointed out that the defect in pyruvate metabolism is certainly present at an early stage in the development of the skin lesion, although no evidence has as yet been obtained that it actually precedes the development of visible oedema, since, by the time respiratory measurements were actually begun, using the skin removed 5 min. after contamination, it might be argued that oedema would also have been present had the skin still been on the animal.

SUMMARY

1. The effect of low concentrations of sodium arsenite and lewisite on the respiration of rat skin slices in the presence of pyruvate and succinate has been studied.

2. Under the conditions used, addition of these arsenicals caused a marked inhibition of the small extra oxygen consumption due to pyruvate (86–100 %), but only a slight effect on succinate oxidation (0–19 %).

3. The respiration of skin contaminated *in vivo* with lewisite has been compared with that of normal skin slices. In the contaminated skin there was again a marked inhibition of pyruvate oxidation but no significant inhibition of succinate oxidation.

4. The significance of these changes to the pathogenesis of vesication is briefly discussed.

Most of the work described here was contained in a report submitted to the Ministry of Supply in 1940 (Thompson). It was carried out as part of a programme of extra-mural research for the Ministry of Supply under the direction of Prof. R. A. Peters, M.C., F.R.S. My thanks are due to the Chief Scientific Officer, Ministry of Supply, for permission to publish, and to Miss M. R. Kempson for technical assistance.

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British Anti-Lewisite

1. ARSENIC DERIVATIVES OF THIOL PROTEINS

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(*Received* 5 *March* 1946)

The basic mechanism by which arsenic injures living cells and exerts its toxic effect on the whole organism, whether protozoal or metazoal, has been for many years the subject of extensive research. To a large degree this has been the outcome of the introduction of organic compounds of arsenic for chemotherapeutic purposes. Subsequently arsenic was introduced for offensive purposes against man, in the form of the arsenical smokes and vesicants, once more focussing interest on the problem of its mode of action and on the prevention or treatment of its effects.

Although arsenic has in the past been described as a 'general protoplasmic poison' it is not an

active protein precipitant. This fact alone suggests an action on the functional activities rather than on the structural basis of living cells. It is natural, therefore, that the action of arsenic on the enzyme systems of cells should have received attention, and it is now known from earlier investigations carried out in this laboratory and elsewhere (Peters, Sinclair & Thompson, 1946; including bibliography of earlier work) that sodium arsenite, lewisite and other arsenicals are highly selective in the toxic effects exerted by them on a number of enzymes. The pyruvate oxidase system in brain, for example, is markedly inhibited by low concentrations of arsenic, whereas the lactate dehydrogenase, the α-glycerophosphate dehydrogenase and the cytochrome oxidase systems of brain, for example, are relatively unaffected by very much higher concentrations. It seemed in fact that the action of arsenic on the enzymes studied showed a certain similarity to the action of iodoacetate, since pyruvate oxidation is also markedly inhibited by this poison (Peters, Rydin & Thompson, 1935), while lactate and α-glycerophosphate dehydrogenases are almost unaffected even after incubation with M/50 iodoacetate for 1 hr. (Hopkins, Morgan & Lutwak-Mann, 1938).

Since the demonstration by Dickens in 1933 that iodoacetate combines with the thiol groups in cysteine and reduced glutathione, results published by many authors have led to the view that whenever iodoacetate inhibits the activity of a biological system it does so by reaction with thiol groups present in that system and necessary for its normal functioning. In direct support of this, from the standpoint of enzyme function, Rapkine (1933) has shown that iodoacetate reacts readily with the thiol groups in proteins.

If this similarity between the actions of iodoacetate and the arsenicals on respiratory enzyme systems is real it might therefore be expected that arsenic also exerts its toxic action by combining with the thiol groups in certain enzymes. The earlier work of Voegtlin and his associates had indeed led them also to the view that the toxic action of arsenoxides on living cells involves a reaction with thiol groups, although these workers were for the most part concerned with small molecular thiols such as reduced glutathione. Thus, in 1923 Voegtlin, Dyer & Leonard showed that the trypanocidal action of 3-amino-4-hydroxyphenylarsenoxide ('arsenoxide') could be counteracted by the addition to the trypanosome suspensions of cysteine, reduced glutathione, or other simple monothiols. Further, the toxic action of arsenoxide in rats could be diminished or even prevented by the intravenous injection of glutathione immediately before the administration of the arsenical (Voegtlin, Dyer & Leonard, 1925). In both trypanosomes and rats, however, a large excess of glutathione was required to abolish the effects of the arsenoxide, a ratio of 10 g.mol. of glutathione to 1 of arsenoxide being necessary, while in the case of sodium arsenite it was found that a ratio of 40 : 1 was required to abolish trypanocidal activity (Rosenthal & Voegtlin, 1930). These workers also showed that the reduction in oxygen consumption of animal tissues caused by arsenoxide could be prevented by the addition to the system of reduced glutathione in a ratio of 10 g.mol. to 1 of arsenoxide (Voegtlin, Rosenthal & Johnson, 1931); but again, when sodium arsenite was used in place of arsenoxide, 40 g.mol. of glutathione were required, a ratio of 10 : 1 being entirely without effect.

More recently this work has been extended by Eagle (1939), who has shown that glutathione is also capable of abolishing the antispirochaetal action of arsenoxide; in this case also a large excess of glutathione was required (GSH : As = 10 : 1); cysteine was found to be less effective, even 12–14 g.mol. failing to abolish the activity entirely.

It seems clear, therefore, that under the various conditions used by these workers an excess of a simple thiol compound, such as glutathione, is able to protect a variety of biological systems against the toxic effects of arsenoxide and to a lesser extent against the effects of sodium arsenite.

That the protection afforded by these simple monothiols is only partial and in some cases inadequate from the physiological standpoint is, however, clearly shown by the work of Schmitt & Skow (1935), who investigated the ability of monothiols to prevent the toxic action of arsenite on the medullated nerves of frogs. They found that while cysteine and reduced glutathione delayed the extinction of the nerve action potential produced by arsenite, in no case were they able to prevent the eventual extinction of the potential, regardless of the ratio SH : As. Nor was it possible by the addition of monothiols to produce any recovery once the action potential had been abolished by arsenite. They also claimed that a large excess of cysteine (150 : 1) was unable to prevent the inhibition of respiration of nerve produced by arsenite.

Similar evidence of partial but inadequate protection was provided by Walker (1928), who reported that sodium thioglycollate had an inhibitory effect on the toxic action of diphenylchlorarsine on glaucoma; and that if monothioethylene glycol was added at the end of 3 min. to a culture that had received a 3 min. lethal dose of the arsenical, rapid revival of the organisms occurred, but was followed by death in 1–2 hr.

Finally, Sinclair (quoted by Peters, 1940) has shown that a variety of monothiols, even when present in large excess (up to 200 equivalents),

failed to protect the brain pyruvate oxidase system from the toxic action of lewisite.

Moreover, the evidence from this work relating the toxicity of arsenic to its reaction with essential tissue thiols is indirect. As early as 1909 Ehrlich had suggested that the 'chemo-receptors' for arsenic might be hydroxyl or thiol groups, but the earliest direct evidence in support of this appears to have been provided by Walker (1924), who, working with Prof. R. A. Peters, showed that the addition of certain arsenicals such as diphenylchloroarsine to dried muscle preparations (Hopkins & Dixon, 1922) containing so-called 'fixed' SH groups (i.e. thiol groups in protein molecules) led to the inhibition of certain catalytic effects shown by these preparations, this inhibition being accompanied by the rapid disappearance of the fixed SH reaction of the muscle powder. In Walker's earliest work there was no proof that the inhibition of the SH reaction was due to combination of the thiol groups with the arsenical, but after the development of his cyanide test for disulphide groups (Walker, 1925) it became clear that after removal of the SH reaction by the addition of the arsenical no recovery was brought about by treatment with cyanide, suggesting that true combination might have taken place.

Although Voegtlin *et al.* (1925) had suggested that in addition to an ability to combine with simple thiols such as glutathione, arsenic might also react with thiol groups present in proteins, it was not until 1932 that Rosenthal demonstrated that trivalent arsenic could not be ultra-filtered from suspensions of arsenic-treated heat-coagulated proteins containing thiol groups. Further, the nitroprusside reaction of these protein suspensions and of washed muscle powder and washed minced liver suspensions was caused to disappear by treatment with the arsenoxides.

In order to investigate further the hypothesis that arsenic, like iodoacetate, exerts its toxic effects by combination with tissue thiols, including 'thiol proteins', it was decided to study *in vitro* the reaction occurring between arsenicals and a soluble thiol protein, and to examine the degree of dissociation at different hydrogen-ion concentrations of any compound formed between the reactants. Ideally, in view of the inhibitory effect of trivalent arsenicals on pyruvate oxidation, and the evidence obtained showing that disorganization of this system exists in the bodies of animals poisoned with arsenic (Peters *et al.* 1946; Thompson, 1946), it would be of the greatest interest to examine the reaction with the isolated purified pyruvate enzyme; unfortunately, this enzyme has not yet been obtained from animal sources in a purified state.

Preparations of urease, myosin and crystalline lens protein were investigated but since, for the work envisaged, it was necessary to have available relatively large amounts of protein, kerateine, a derived protein, obtained by rupture of the disulphide bonds of keratin, was finally chosen.

The general procedure adopted in the experiments about to be described was to add the arsenical to buffered solutions of kerateine until the nitroprusside reaction of the solution disappeared. The protein was then precipitated at pH 4·6 with acetic acid, washed and dried. The nitrogen, sulphur and arsenic contents of the derivatives were then estimated, and the stability of the proteins investigated by analysis after re-precipitation or dialysis.

METHODS

Analyses. Nitrogen analyses were carried out in duplicate on the various preparations by the method of Conway & Byrne (1933); sulphur by the macro-Carius method. Arsenic was estimated by the titrimetric micro-method of Allcroft & Green (1935). After digestion of the material with perchloric and nitric acids, the subsequent reduction to arsine was carried out by immersion of the generating flask in a water-bath at 80–100°. Using 5 ml. of N/75-$AgNO_3$ in each of the three absorption towers, 97–100% recovery of amounts of arsenic varying from 50–300 μg. was obtained.

Preparation of kerateine. Kerateine was prepared from washed human hair by treatment with alkaline cyanide at 38° according to the method of Goddard & Michaelis (1934). The solution obtained by this treatment was filtered, precipitated at pH 4·6 with glacial acetic acid, washed with dilute acetic acid, and either dried or redissolved in buffer at pH 7·0 and used directly for the preparation of arsenical derivatives.

In contrast to the preparations obtained by Goddard & Michaelis from wool, the dried kerateine from human hair was soluble in water and neutral salt solutions. The solutions gave a strong nitroprusside reaction. The nitrogen and sulphur contents of three different preparations are given in Table 1.

Table 1. *Nitrogen and sulphur content of kerateine from human hair*

N (%)	S (%)
14·9	1·37
13·9	1·17
14·9	1·39

RESULTS

Arsenic derivatives of kerateine. The preparation of the lewisite (chlorovinyldichloroarsine) derivative of kerateine was carried out as follows: kerateine derived from 50 g. hair was precipitated at pH 4·6, filtered off and redissolved in 200 ml. of 0·4 M-phosphate buffer (pH 7·0). The solution was then titrated with 19% (w/v) lewisite in ethanol with vigorous stirring until the nitroprusside reaction in dilute ammonia became negative. At the end of the titration the solution was clear, and showed no

evidence of any precipitation of lewisite oxide; the pH was maintained at 7·0 throughout the addition of the arsenical. Glacial acetic acid was then added until the precipitation of the protein was complete (pH 4·6). The precipitate was then filtered off, washed with water and dried.

The arsenite derivative was prepared in a similar manner, 4·95 % (w/v) neutralized sodium arsenite being used to titrate the kerateine solution. A large excess of sodium arsenite was required to abolish the nitroprusside reaction, and in some preparations the nitroprusside reaction never completely disappeared. One preparation of the ethyldichloroarsine derivative was also made and analyzed.

The analytical figures on the various dry preparations obtained by the treatment of fresh solutions of kerateine with the arsenicals are shown in Table 2.

Table 2. *Analysis of arsenic derivatives of kerateine*

Derivative	N (%)	S (%)	As (%)	
Lewisite	—	1·36	0·58	
	—	—	0·48	
	15·6	1·32	0·63	Mean As = 0·52 %
	14·8	1·41	0·46	
	14·8	1·31	0·47	
	—	—	0·53	
Ethyldichloroarsine	—	—	0·52	
Sodium arsenite	15·2	1·49	0·56	
	13·5	1·25	0·33	Mean As = 0·41 %
	14·8	1·62	0·46	
	14·2	1·26	0·30	

It will be seen that treatment of kerateine with lewisite or arsenite yields derivatives containing approximately 0·5 and 0·4 % arsenic respectively. The arsenic content of the derivatives was found to be independent of the amount of arsenic used to titrate the kerateine solution; thus, in the case of the lewisite derivatives, amounts varying from 0·6 to 3·9 ml. of 19 % (w/v) lewisite per g. kerateine were used in the different preparations, i.e. from 8 to 55 times the amount of arsenic found in the protein. All the proteins prepared gave clear solutions below pH 4 and above pH 5·4, and showed maximal flocculation at pH 4·6.

The stability of these arsenic-kerateine derivatives was next investigated by determining the arsenic contents after reprecipitation at pH 4·6, the precipitate being once more washed and dried, and after dialysis against distilled water at room temperature for 20 and 44 hr. The results of these experiments are shown in Table 3.

It will be seen that the lewisite derivative is very much more stable to reprecipitation or dialysis than the arsenite derivative, 84–87 % of the arsenic

Table 3. *Arsenic content of kerateine derivatives after reprecipitation or dialysis*

Derivative	Original As (%)	As after one reprecipitation (%)	As after 20 hr. dialysis (%)	As after 44 hr. dialysis (%)
Lewisite	0·63	0·53 (84 %)	0·35 (56 %)	0·30 (48 %)
Lewisite	0·46	0·40 (87 %)	0·30 (65 %)	0·21 (46 %)
Arsenite	0·56	0·17 (30 %)	0·07 (13 %)	—
Arsenite	0·33	0·18 (55 %)	0·04 (12 %)	—
Arsenite	0·46	0·20 (44 %)	0·09 (19 %)	—

(The numbers in brackets give the percentage remaining of the original arsenic content.)

remaining attached to the protein after reprecipitation in the case of the lewisite-kerateine, as compared with only 30–55 % in the case of the arsenite-kerateine. The dialyzed samples show an even more striking difference between the two derivatives.

Goddard & Michaelis (1935) have shown that kerateine can readily be oxidized from the thiol to the disulphide form at neutral reaction by atmospheric oxygen: they have called this disulphide form metakeratin, and have pointed out that in spite of the reformation of disulphide bonds in the molecule the protein is still soluble and its isoelectric point is unchanged. In view of this ready transformation from the thiol to the disulphide form without any other apparent change in the molecule or its physical properties, solutions of metakeratin have also been treated with the same amounts of lewisite and arsenite that were used in the preparation of the corresponding kerateine derivatives. Such an experiment should help to decide whether the arsenic contained in the kerateine derivatives is present in true combination with the thiol groups or merely non-specifically attached to the protein molecule and precipitating with it.

The metakeratin was prepared by passing oxygen through solutions of kerateine until the nitroprusside reaction had completely disappeared. After treatment with the arsenicals the metakeratin was then precipitated at pH 4·6, washed and dried in the same manner as was used for the kerateine derivatives. The analytical figures obtained on the dried preparations and after a further reprecipitation at pH 4·6, are given in Table 4.

Table 4. *Analysis of metakeratin after treatment with lewisite, arsenite and arsenate*

Derivative	S (%)	As (%)	As after one reprecipitation (%)
Lewisite	1·22	0·04	0·02
Lewisite	1·37	0·07	—
Arsenite	1·48	0·09	0·03
Arsenate	1·13	0·04	0·04

A further experiment was carried out in which the kerateine was incompletely oxidized, the final solution used for the titration with lewisite giving a very faint nitroprusside reaction. The arsenic content of this preparation was 0·12 %.

Treatment of metakeratin with arsenicals therefore results in the formation of derivatives containing only a small fraction of the arsenic present in the corresponding kerateine derivatives. In order to show that the small amount of arsenic present in these lewisite and arsenite derivatives was probably due to adsorption, an experiment was carried out using sodium arsenate as the reagent, since, if no thiol groups are present, this cannot be reduced to the reactive trivalent form, and any arsenic present in the derivative would therefore most likely be due to adsorption. It will be seen that, taking into account experimental error, no significant difference exists between the arsenate and the other derivatives.

Since it has been shown by Cohen, King & Strangeways (1931) that the combination of pentavalent arsenicals with thiols takes place according to the equation

$$R\mathrm{AsO_3H_2} + 4R^1\mathrm{SH} \rightarrow R\mathrm{As}(\mathrm{S}R^1)_2 + R^1\mathrm{S{-}S}R^1 + 3\mathrm{H_2O},$$

it was anticipated that if kerateine was treated with sodium arsenate the amount of arsenic found in the product would be intermediate between those of the arsenite-kerateine and arsenate-metakeratin.

The reaction between sodium arsenate and kerateine was therefore carried out under the same conditions as for the arsenite-derivative, and, as will be seen from Table 5, the products had the expected intermediate arsenic contents.

Table 5. *Analysis of kerateine treated with excess of sodium arsenate*

N (%)	As (%)	S (%)
14·1	0·19	—
14·9	0·11	1·31
14·3	0·06	1·30

Mean As = 0·12 %.

These results still further support the view that the arsenic present in the lewisite and arsenite-kerateine derivatives is present in true combination with the thiol groups and not associated non-specifically with the protein.

If this is true, the arsenic content of these derivatives should be dependent on the thiol content of the parent proteins. A variety of compounds (I_2, $K_3Fe(CN)_6$, phenylmercuric chloride, dichlorophenolindophenol and cystine) has been used in the past for the estimation of thiol groups, but none has given entirely satisfactory results. More recently Kuhn & Desnuelle (1938) and Greenstein (1938) have suggested porphyrindin as a more suitable reagent. This was, therefore, synthesized according to the method of Kuhn & Franke (1935), and was standardized against cysteine ester hydrochloride. It was found necessary to use carefully controlled conditions in order to avoid over-titration, and these conditions were rigidly adhered to both in the standardization against cysteine ester and the titration of the protein solutions. From these titrations it was possible to calculate the amount of arsenic which would combine with the protein, assuming the ratio of 1 As : 2 SH. The lewisite derivative was next formed from a sample of the protein solution; after washing and drying in the usual way the arsenic content of the dry preparation was estimated.

Table 6 shows the arsenic content as found and as calculated from the porphyrindin titration in four different preparations.

Table 6. *Estimated* As *content and content calculated from porphyrindin titration* (*lewisite-kerateine*)

	Found (%)	Calc. from thiol titration (1 As : 2 SH) (%)
	0·49	0·45
	0·63	0·46
	0·58	0·47
	0·60	0·46
Mean	0·58	0·46

From the results of the four experiments shown in Table 6 it will be seen that, when calculated from the porphyrindin titration of the thiol groups originally present in the parent kerateine on the assumption that 1 mol. of lewisite combines with two thiol groups, the mean arsenic content of the lewisite-kerateine compound would be 0·46 %. The arsenic content as determined by analysis in these four experiments ranged from 0·49 to 0·63 %. The value calculated for the ratio 1 As : 2 SH accounts therefore for 73–92 % of the arsenic actually found.

It must be realized of course that since the experimental conditions involved an excess of lewisite it was possible that some of the molecules of lewisite may have reacted with only one thiol group so that the ratio 1 As : 2 SH would not account for all the arsenic found.

DISCUSSION

The main evidence for Voegtlin's view that the toxic action of arsenic is due to its combination with thiol groups is based on the ability of simple thiols, when present in excess, to protect certain systems from the effects of certain arsenicals. This protection, however, is not found in all cases, and therefore

such evidence cannot be held to be conclusive. The explanation of this inconsistency possibly lies in the dissociability of the As—S link (Cohen *et al.* 1931; Strangeways, 1937) since it has been shown that arsenoxides and thiols react thus:

$$RAsO + 2R^1SH \rightleftharpoons RAs(SR^1)_2 + H_2O.$$

The final equilibrium established depends on the hydrogen-ion concentration, acid shifting it to the right and alkali to the left. The equilibrium can also be altered by the addition of a second thiol whose affinity for arsenic is greater than that of the first. Such an explanation may account for the high toxicity of tricysteinylarsine (Voegtlin *et al.* 1931) which has been shown to have as great a toxicity as arsenite on the respiration of living cells, the thiol groups of some essential enzymes, by reason of a possible high affinity for arsenic, combining perhaps with the arsenite released by hydrolysis from the tricysteinylarsine, thus driving the reaction to the left.

It seemed important therefore to investigate the nature of the interaction occurring between arsenic and thiol groups in proteins. Owing to the impracticability of obtaining, at the time, adequate amounts of an arsenic-sensitive enzyme in a purified state kerateine was chosen as a model; it is fully realized that this was not an ideal protein for the investigation in view, but it was felt that it would provide a suitable thiol protein for initial experiments towards this end. So far as we are aware no arsenical derivatives of soluble proteins have yet been prepared under physiological conditions. The nearest approach to such an investigation is that of Wölfing & Möller (1935), who treated acid hydrolysates of keratin with hydroxyphenylarsenoxide. These hydrolytic products, however, were of high sulphur content and proteose in nature. We also have obtained preparations with high sulphur and arsenic contents (As, 4·0%; S, 5·65%) by using the pH 4·6 filtrate, but these contained proteoses and smaller molecular compounds, and hence were abandoned as being unsuitable for our purpose.

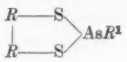
The experiments described above are interpreted as showing that under physiological conditions of temperature and hydrogen-ion concentration lewisite and arsenite combine with kerateine to give water-soluble protein derivatives, lewisite forming a more stable compound than arsenite. The arsenicals combine solely with the thiol groups of the kerateine. The evidence for concluding that the arsenic is present in these derivatives in true combination with the thiol groups, and is not merely present in non-specific physical association with the proteins, is as follows:

(1) Treatment of kerateine with lewisite or arsenite results in the formation of compounds whose arsenic content is independent of the amount of arsenic added.

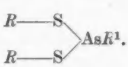
(2) The arsenic content of the derivatives bears a direct relationship to the thiol content of the parent protein.

(3) The thiol content of kerateine, as determined by porphyrindin titration, is sufficient to account for all the arsenic found in the derivatives.

The lewisite appears to be relatively firmly combined with the protein, as judged by the arsenic content after reprecipitation and dialysis. Moreover, from the porphyrindin titrations it appears that at least 75% of the arsenic found in the lewisite-protein is in combination with two thiol groups. In view of the ease with which kerateine can be re-oxidized to the disulphide form it seems probable that the arsenic has combined with two SH groups closely placed in space on the same molecule, to form a ring. On physico-chemical grounds ring compounds of the type



would be expected to be more stable than compounds of an arsenical with 2 mol. of a monothiol, i.e. of the type



There emerged from these experiments therefore the conception that the high toxicity of trivalent arsenicals might be due to their ability to combine with two closely situated essential SH groups on certain tissue proteins to form relatively stable arsenical rings. If this conception is correct it follows that efficient protection against these arsenicals would only be afforded by the presence of a competing dithiol capable of forming a ring compound at least as stable as the compound of arsenical with the tissue 'acceptor'. An account of the further development of this theory, and of the elaboration of a therapeutically successful dithiol, is given in succeeding papers. A brief, preliminary review of the whole work has already appeared (Peters, Stocken & Thompson, 1945).

SUMMARY

1. The preparation of derivatives of kerateine with lewisite, ethyldichloroarsine, sodium arsenite and sodium arsenate is described.

2. The stability of these arsenical proteins has been investigated.

3. It appears that under the conditions used in this work arsenic combines with proteins by reacting with the thiol groups, and no evidence has been

obtained that any significant combination occurs with other groups in the kerateine molecule.

4. At least 75% of the arsenic found in the lewisite-derivative is in combination with two thiol groups, and the hypothesis has consequently been put forward that dithiols may prove more effective antidotes to arsenic than the mono-thiols used in the past.

This work was carried out as part of a programme of extra-mural research for the Ministry of Supply under the direction of Prof. R. A. Peters, M.C., F.R.S., and was communicated to the Ministry in 1940 (Stocken & Thompson). We wish to express our gratitude to Prof. N. V. Sidgwick, F.R.S., and Mr J. St L. Philpot for their helpful advice, and to the Chief Scientific Officer, Ministry of Supply for permission to publish the facts. Our thanks are also due to Mr C. Dear and Mr E. Facer for skilled technical assistance.

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British Anti-Lewisite

2. DITHIOL COMPOUNDS AS ANTIDOTES FOR ARSENIC

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(*Received* 5 *April* 1946)

In a previous paper the preparation of arsenic derivatives of kerateine has been described (Stocken & Thompson, 1946). In the course of that work it was found that the arsenicals had combined with the SH groups present in the kerateine, and that at least 75% of the arsenic in the lewisite derivative was present in the proportion 1 As : 2 SH. It seemed probable therefore that the arsenic had combined with 2 SH groups closely placed in space on the same molecule to form a ring compound, and it was suggested that efficient protection against trivalent arsenicals would only be afforded by the presence of a dithiol capable of forming a relatively stable ring compound with the arsenical, instead of the much more dissociable 'open chain' compounds formed between arsenic and the monothiols investigated in the past.

Much of the earlier work (Voegtlin, Dyer & Leonard, 1923, 1925; Walker, 1924; Rosenthal & Voegtlin, 1930; Voegtlin, Rosenthal & Johnson, 1931) demonstrating the limited capacity of simple monothiols to protect certain biological systems from the toxic action of the therapeutic arsenicals was also reviewed, but it was pointed out that the protection afforded by compounds of this type was much less complete when exerted against sodium arsenite than against the therapeutic arsenoxides, and that in some cases the protection was inadequate from the physiological standpoint (Walker, 1928; Schmitt & Skow, 1935).

In the case of lewisite, experiments carried out in this laboratory have shown that, of an extensive series of monothiol compounds studied, none of the substances investigated has been able to protect against lewisite. Thus, Sinclair (quoted by Peters, 1940) found that the brain pyruvate oxidase system was not protected against lewisite or arsenite by very large concentrations (a ratio of 1 : 200 mol.) of glutathione or cysteine.

The contrast between these results and those of the earlier workers with aromatic therapeutic arsenoxides probably depends largely on differences in the degree of dissociation of the thioarsinites formed by interaction of these arsenicals with the thiol. In the case of the aromatic arsenoxides Cohen, King & Strangeways (1931) were able to demonstrate that compounds with simple monothiols undergo extensive dissociation in 0·1N-NaOH, while in sodium bicarbonate solution several of the thioarsinites studied gave a feebly positive nitroprusside reaction, indicating a slight degree of dissociation. It must be remembered also that in the various biological systems studied by Voegtlin a large excess of the thiol was necessary to counteract the toxic effects of the arsenoxide.

More recently, Strangeways (1937) has investigated the toxicity to trypanosomes *in vitro* of three thioarsinites, including the 'arsenoxide'-glutathione compound, and has shown that the lethal activities of equimolar solutions of this thioarsinite and of its parent arsenoxide are identical. Moreover, using the dilution of arsenoxide employed by Voegtlin *et al.* (1923), she confirmed their observation that complete inhibition of toxic action could be obtained with a ratio of 1 : 10 mol. of glutathione, but found that on using more dilute solutions of arsenoxide the protection afforded by 10 mol. of glutathione became less complete, until at high dilutions no protection could be observed at all: in strong solutions the excess of glutathione favours the formation of the thioarsinite, whereas more dilute solutions tend to bring about hydrolysis with reliberation of the toxic arsenoxide.

It is clear, therefore, that the 'arsenic acceptor' in living cells must form a more stable compound with arsenic than do any of the simple monothiols so far investigated. For the reasons already given it was decided therefore to study the protection afforded by simple dithiol compounds.

Two considerations guided us in our choice of dithiols: first, since on physico-chemical grounds it was desirable to form 5- or 6-membered ring compounds between the arsenical and the thiol, investigations were begun with 1:2- and 1:3-dithiol compounds; secondly, since the work was designed primarily to find an antidote to the vesicant action of lewisite, rapid penetrability of human skin was desirable, necessitating the initial choice of simple small molecular dithiols. Philpot (private communication) in a related research in this laboratory had shown that short-chain fatty acids penetrate the skin more rapidly than long-chain compounds.

The first dithiol to be used was toluene-3:4-dithiol ('Dithiol', British Drug Houses Ltd. Spot Test Reagent) and although encouraging results were obtained with an enzyme as the test system, the local irritant action of the compound became apparent when it was applied to the skin of rats contaminated with lethal amounts of lewisite, and though death was prevented it seemed that aromatic thiols, like phenols, are capable of exerting a local necrotizing effect, and hence were unsuitable.

Ethane-1:2-dithiol was next prepared and investigated, but was considered impractical as an anti-lewisite agent under field conditions on account of its high volatility.

Since a water-soluble compound of low volatility, and possibly also of high viscosity, was indicated, 2:3-dimercaptopropanol was next prepared in the hope that its chemical similarity to glycerol might also extend to such properties as volatility and ready skin penetrability. The results of preliminary tests with this compound, later called BAL (or British Anti-Lewisite) by the Americans, suggested that it would be suitable for more extended investigations. *In vivo* tests with animals, and later human tests, were therefore undertaken. In the course of these tests certain other dithiol compounds were prepared and their antidotal activity investigated.

The greater part of the work described here was carried out in 1940 and was reported to the Ministry of Supply in 1941 (Stocken & Thompson). Much of it has since been confirmed and extended both in this country and in the United States. No attempt has been made here to review this later work.

Six monothiols and 12 dithiols have been studied in the course of the work described here. The details of the preparation and properties of these and other thiols are given in a separate communication (Stocken, 1946).

EXPERIMENTAL AND RESULTS

The reaction of trivalent arsenicals with mono- and dithiols

In a previous report (Stocken & Thompson, 1940) it was shown that the addition of lewisite to solutions of kerateine caused a disappearance of the nitroprusside reaction at pH 9·0. This finding was in contrast to the results obtained by Cohen *et al.* (1931), who found that the thioarsinites formed by interaction of arsenoxides with simple monothiols were extensively dissociated in alkaline solution. It

was of interest therefore, in the first place, to determine whether simple dithiols reacted with lewisite to form alkali-stable compounds, similar to the lewisite-kerateine derivative. Briefly, it was found that the addition of equivalent amounts of 'lewisite oxide' (chlorovinylarsenoxide) or of sodium arsenite to solutions of BAL resulted in a complete disappearance of the nitroprusside reaction. The previous observations of Cohen *et al.* (1931) were confirmed by the use of 2-mercaptoethanol.

Experiments were next carried out to discover the relative rates of hydrolysis of thioarsinites prepared by reacting lewisite with monothiols, dithiols and thiolproteins respectively. For this purpose, in addition to kerateine, an acetone-dried rabbit muscle powder and crude crystalline lens protein prepared from ox eyes by a modification of the method of Woods & Burky (1927) were used.

One equivalent of lewisite oxide was added to one equivalent of the thiol in 0·2 M-phosphate buffer, pH 7·3; half an equivalent of porphyrindin was added next and the decolorization times noted. The final concentration of the thioarsinite was the same in all cases. The reactions are:

(1) $R\text{—}S\diagdown\ \diagup\text{AsCH : CHCl} + H_2O \rightleftharpoons \text{ClCH : CHAsO} + 2R\text{SH}$
$R\text{—}S\diagup$

(2) $2R\text{SH} + \text{Porphyrindin} \rightarrow R\text{—S—S—}R + \text{Leucoporphyrindin}$

In the case of the thiol proteins the SH content was first determined by porphyrindin titration and the end-point confirmed by the nitroprusside reaction. In the absence of lewisite all the thiols included in Table 1 titrated rapidly with porphyrindin, with the exception of monothioethylene glycol which took 1½ min.; lewisite itself does not decolorize porphyrindin. The results given in Table 1 show that there is a very marked difference in the decolorization times given by thioarsinites derived from monothiols and dithiols, and that those for the protein derivatives are slightly longer than for monothiols. If it be assumed that the rates of formation of the thioarsinites are comparable, these decolorization times should give a measure of the rates of hydrolysis of these compounds. From this it may be concluded that at equilibrium in a system containing equivalent amounts of lewisite oxide and a monothiol, a dithiol and a thiol protein, the amount of lewisite combined with the dithiol will be much greater than that combined with the protein which in turn will be slightly greater than that combined with the monothiol.

Table 1. *Hydrolysis rates of thioarsinites prepared from lewisite plus monothiols, dithiols and thiol proteins*

(Expressed as time taken to decolorize 0·5 equiv. of porphyrindin.)

Thiol	Decolorization time (min.)
Cysteine	0·25
Thiolacetic acid	0·5
Aminothiophenol	0·83
Glutathione	2·5
2-Mercaptoethanol	3·5
Protein from muscle powder	3·0
Lens protein	3·5
Kerateine	6·0
2:3-Dimercaptopropanol (BAL)	>180
Ethane-1:2-dithiol	>180
1:3-Dimercaptopropanol	>180
Propane-1:3-dithiol	>180
ββ′-Dimercaptodiethylether	>180

An attempt was also made to measure the decolorization times of ethanethiol, pentane-1:5-dithiol and ββ′-dimercaptodiethyl sulphide in the presence and absence of lewisite; it was found, however, that these compounds by themselves decolorized porphyrindin only very slowly, so that no conclusion could be drawn from the reaction of their derived thioarsinites with porphyrindin. This may be due to these compounds having higher redox potentials than the others cited.

Effectiveness of dithiols in protecting the brain pyruvate oxidase system from the toxic effects of arsenic

The principle involved in these tests has been the measurement of the ability of dithiol compounds to abolish, or diminish, the inhibition of pyruvate oxidations in brain produced by the presence of small amounts of lewisite oxide. The pyruvate oxidase system was chosen for these initial biological tests, as it had been previously shown (Krebs, 1933; Peters, 1936; Sinclair & Thompson, 1940) that this enzyme system is highly sensitive to arsenical poisoning, and provides therefore a convenient and rapid biochemical method for determining the effectiveness of water-soluble compounds as antidotes for arsenic. It is interesting to note that the results obtained with this *in vitro* test were strikingly confirmed by the *in vivo* tests described later in this paper.

Pigeon brain *brei* respiring in Ringer-phosphate solution at pH 7·3 and at 38° was used throughout. The earlier experiments were carried out with neutralized lewisite oxide, the final concentrations used (0·016 mM) being sufficient to cause approximately 50 % inhibition of the total respiration. The thiols were dissolved in a small volume of dilute alkali, and were brought to the required concentration with phosphate buffer, so that the pH of the respiring medium was not detectably altered by their addition. The oxygen consumption of the system was measured manometrically over 3 successive half-hour periods.

Table 2 shows the toxicity to the pyruvate oxidase system of the various compounds investigated, the figures representing the percentage inhibition of the total respiration of the brei in the presence of 0·02 M-sodium pyruvate. It will be seen that the aliphatic dithiol compounds investigated are strikingly non-toxic to this enzyme system. The addition of the dithiol in most cases resulted in a small increase (5–15 %) in oxygen uptake. The greater toxicity of toluene-3:4-dithiol may have been due in part to the fact that the sample used had undergone partial oxidation.

Table 2. *Toxicity of dithiol compounds to the brain pyruvate oxidase system*

Compound	Concentration (mM)	Inhibition of O_2 uptake (%) 0–30 (min.)	30–60 (min.)	60–90 (min.)
Toluene-3:4-dithiol	0·21	19	25	26
Ethane-1:2-dithiol	0·36	0	0	0
2:3-Dimercaptopropanol	0·27	0	2	0
1:3-Dimercaptopropanol	0·27	0	0	0
Propane-1:3-dithiol	0·31	0	0	4
$\alpha\beta$-Dimercaptopropionic acid	0·48	6	0	0

The effect of these dithiols in reducing the toxicity of lewisite oxide to the pyruvate oxidase system was next investigated. In each experiment the thiol was added to the system immediately before the addition of the lewisite; flasks were also set up in which the thiol was added to the unpoisoned enzyme system in order to control the small extra oxygen uptake frequently induced by the presence of the thiol. Table 3 gives a comparison of the percentage inhibition of oxygen uptake produced by lewisite oxide in the absence and presence of various thiol compounds.

The above results indicate that all the dithiol compounds here investigated either markedly diminish or completely abolish the inhibition produced by lewisite oxide. Moreover, this effect is present in dilute solutions of the thiols; thus, in the case of BAL a very marked reduction in the toxicity of lewisite is brought about by a ratio of 1 : 4 mol. of dithiol, a ratio of 1 : 8·5 mol. completely abolishing it.

In view of the original *in vitro* work on the reaction between lewisite and kerateine, an experiment was carried out to determine whether any antidotal activity was exhibited by this thiol protein. A solution of kerateine, prepared from hair by the method of Goddard & Michaelis (1934), was therefore made up to give a final concentration of 0·3 % in the Warburg flasks; the toxicity of lewisite was much reduced in the presence of this concentration, thus showing that kerateine also can compete

Table 3. *Antidotal effect of thiol compounds on the toxicity of lewisite oxide to pyruvate oxidation*

Compound	Concentration (mM)	Inhibition of O_2 uptake (%) Lewisite oxide (0·016 mM) 0–30 (min.)	30–60 (min.)	60–90 (min.)	Lewisite oxide (0·016 mM) + thiol 0–30 (min.)	30–60 (min.)	60–90 (min.)
Toluene-3:4-dithiol	0·02	67	59	69	0	1	10
	0·02	63	61	72	3	21	27
	0·21	67	59	69	0	0	0
Ethane-1:2-dithiol	0·07	56	53	50	7	2	4
	0·36	56	53	50	0	0	0
2:3-Dimercaptopropanol (BAL)	0·05	72	46	47	—	18	15
	0·14	47	47	40	0	3	0
	0·27	72	46	47	0	0	0
	0·27	51	46	46	0	3	0
1:3-Dimercaptopropanol	0·05	45	47	42	12	20	23
	0·27	45	47	42	0	2	2
Propane-1:3-dithiol	0·06	49	52	50	4	6	4
	0·31	49	52	50	0	0	0
$\alpha\beta$-Dimercaptopropionic acid	0·10	53	52	54	11	10	9
	0·48	53	52	54	4	6	0
2:3-Dimercaptopropylamine HCl	0·04	61	61	54	68	64	60
	0·27	45	48	49	0	9	17
	0·21	61	61	54	21	20	16
Kerateine (0·3 %)	—	65	52	54	10	8	17
2-Mercaptoethanol	0·09	57	55	—	54	55	—
	0·43	57	55	—	45	64	—
Cysteine hydrochloride	0·14	48	56	38	60	58	40
	0·27	48	56	38	51	56	45

with the pyruvate oxidase system as an 'arsenic receptor'.

The contrast between two monothiols and these dithiols as regards protection of this system against lewisite oxide is also shown; 0·27 mM-cysteine hydrochloride and 0·42 mM-monothioethylene glycol each completely failed to protect against the effects of 0·016 mM-lewisite oxide. The close chemical relationship of 2-mercaptoethanol to ethane-1:2-dithiol and the disparity between these two compounds as antidotes against lewisite oxide is particularly striking.

it seemed desirable to obtain information concerning the stability of the lewisite-dithiol compound. An attempt was made to prepare the compound of lewisite and BAL and to measure its toxicity directly; owing to the extreme insolubility of the compound, however, such a method did not seem easily practicable. It was decided therefore to study this point indirectly by determining the degree of inhibition of pyruvate oxidation produced by adding samples of a brain *brei* to a Ringer-phosphate-pyruvate solution containing equivalent amounts of lewisite and BAL that had been allowed

Table 4. *Effect of 2:3-dimercaptopropanol (BAL) on the toxicity of other arsenical compounds to the pyruvate oxidase system*

	Concentration		Inhibition of O_2 uptake (%)					
			Arsenical			Arsenical + BAL		
Arsenical	As (mM)	BAL (mM)	0–30 (min.)	30–60 (min.)	60–90 (min.)	0–30 (min.)	30–60 (min.)	60–90 (min.)
Phenylarsenoxide	0·015	0·05	52	49	50	3	10	7
	0·015	0·27	52	49	50	3	5	0
Sodium arsenite	0·034	0·27	44	41	42	0	0	2
	0·068	0·27	61	56	65	6	13	17
Ethylarsenoxide	0·016	0·14	41	31	—	0	0	—
Methylarsenoxide	0·016	0·14	36	28	—	0	0	—

Phenyl-, ethyl- and methylarsenoxides, obtained by solution and subsequent neutralization of the corresponding dichlorarsines in water, and neutralized sodium arsenite, have also been used to poison the pyruvate system, and the ability of BAL to prevent this poisoning investigated. The experiments were carried out in the same way as those with lewisite described above, and the results are given in Table 4. It will be noticed that in the majority of the above experiments the diminution in toxicity of the arsenical caused by the dithiol remains approximately constant throughout the three half-hour periods studied. In this connexion

to stand at room temperature (in the experiments already described the lewisite was added to flasks already containing both thiol and brain).

Lewisite (10 μg. as the oxide) was therefore added to a series of Warburg flasks containing Ringer-phosphate solution; 1 equiv. of BAL (in one experiment 1, 2 and 4 equiv.) was then added to each of a pair of bottles, another pair serving as control; after standing for 20–30 min. at room temperature the brain brei was added, and the rate of pyruvate oxidation measured. It will be seen (Table 5) that whereas 10 μg. of lewisite produced 50 % inhibition of activity, a mixture of 10 μg.

Table 5. *Toxicity of lewisite-thiol mixtures (in equivalent proportions) to the pyruvate oxidase system*

		Inhibition of O_2 uptake (%)					
		Lewisite			Lewisite + thiol		
Thiol	No. equiv. of thiol	0–30 (min.)	30–60 (min.)	60–90 (min.)	0–30 (min.)	30–60 (min.)	60–90 (min.)
2:3-Dimercaptopropanol (BAL)	1	51	51	51	6	4	6
	1	52	50	38	4	2	0
	1	73	70	69	0	0	0
	1	57	54	57	0	0	0
	2	51	51	51	0	0	4
	2	52	50	38	3	5	0
	4	51	51	51	0	2	7
$\beta\beta'$-Dimercaptodiethyl sulphide	1	52	50	38	55	68	60
	2	52	50	38	38	47	49
Pentane-1:5-dithiol	1	57	54	57	23	21	20
n-Heptane thiol	1	73	70	69	61	70	64
	1	57	54	57	44	40	45

lewisite with 1 equiv. of BAL (6 μg.) was almost non-toxic to the enzyme system. Moreover, although the measurement of oxygen uptake was continued for 2 hr., there was no indication of any significant increase in the toxicity of the lewisite-BAL compound.

The activities of two dithiols of a different type (which could form 8- or 16-membered rings with the arsenical) and of a monothiol, heptane thiol, were also studied by this method; this latter compound was prepared and used here in order to determine whether a longer-chain, lipid-soluble monothiol that would form a water-insoluble thioarsinite would show any greater antidotal activity than the other monothiols used in this work. It will be seen that one equivalent of *n*-heptane thiol or of $\beta\beta'$-dimercaptodiethyl sulphide were without significant effect, while one equivalent of pentane-1:5-dithiol showed only moderate activity. From these tests too it would seem therefore that 1:2- (or 1:3-) compounds are the most effective types of dithiol for this purpose.

A few experiments have also been done in order to determine whether it is possible by the addition of a dithiol to reverse the inhibition of a pyruvate oxidase system already poisoned by lewisite oxide. As in the previous experiments 0·016 mM-lewisite oxide was used to poison the system. The dithiol (BAL) was added to the side-bulbs of Warburg flasks, and was tipped into the main compartment 15 or 45 min. after poisoning the system with lewisite oxide. Table 6 summarizes the results of these experiments.

If the BAL is added only 15 min. after the commencement of the poisoning, complete reversal of the enzyme inhibition is ultimately obtained; if the addition of the BAL is delayed until the system has been poisoned for 45 min. only about 50% reactivation is effected at the end of a further 1½ hr. by the concentrations of BAL used. It is possible that a larger excess of BAL might bring about more complete reactivation of the system after poisoning for 45 min.

As indicated previously by the results of earlier workers, enzyme inhibition by arsenic does not, at any rate in the earlier stages, appear to be an irreversible phenomenon, and the addition of a dithiol to the brain pyruvate oxidase system as late as 45 min. after poisoning with lewisite can markedly reverse the toxic effects.

Antidotal activity of thiols against lewisite inhibition of respiration of skin

Since the primary objective was the production of an antidote capable of preventing vesication of skin by lewisite and other chloroarsine derivatives, the effectiveness of BAL as an antidote against the lewisite inhibition of the respiration of skin was also studied.

As in experiments described elsewhere (Thompson 1946) slices of young rat skin were used, and the respiration measured at pH 7·3 and at 38° over periods of 1 hr. in the presence of added sodium pyruvate (0·02 M). The results of 2 experiments are shown in Table 7. Two monothiols, 2-mercaptoethanol and cysteine, were also studied.

Table 6. *Ability of 2:3-dimercaptopropanol (BAL) to reverse the toxic effect of lewisite oxide on the brain pyruvate oxidase system*

		Inhibition of O_2 uptake (%)								
		Lewisite				Lewisite + BAL				
Time of contact with poison before BAL tipped in (min.)	Concentration BAL (mM)	15–45 (min.)	45–75 (min.)	75–105 (min.)	105–135 (min.)	0–15 (min.)	15–45 (min.)	45–75 (min.)	75–105 (min.)	105–135 (min.)
15	0·27	51	46	46	—	—↓	1	0	0	—
15	0·27	44	59	43	—	—↓	14	4	0	—
45	0·14	47	47	40	—	—	56↓	36	16	—
45	0·14	64	52	69	60	—	59↓	34	31	28
45	0·14	56	57	53	52	—	54↓	50	23	19
45	0·27	56	57	53	52	—	49↓	48	26	27

(↓ = BAL tipped into system.)

Table 7. *Antidotal effects against lewisite inhibition of respiration of rat skin slices*

(Concentration lewisite = 0·03 mM)

			Inhibition of total O_2 uptake in presence of pyruvate (%)		
		Thiol (mM)	0–60 (min.)	60–120 (min.)	120–180 (min.)
1	Lewisite	—	42	55	61
2	,,	—	58	76	79
1	Lewisite and BAL	0·27	12	13	6
2	,, ,,	0·27	0	4	1
1	Lewisite and 2-mercaptoethanol	0·54	55	60	22
2	Lewisite and cysteine	0·54	48	71	78

As with pyruvate oxidation in brain it will be seen that under the conditions described above,

BAL causes almost complete protection against the effects of lewisite in skin respiration, whereas cysteine and 2-mercaptoethanol, in equivalent concentrations, completely failed to protect. The significance of this finding is emphasized by the results obtained with BAL and 2-mercaptoethanol in the prevention of vesication of human skin by lewisite described in a later section.

Prevention of poisoning by lewisite in rats by dithiols

It was decided to determine next whether BAL or other dithiols are capable of preventing the death of rats following the application to the skin of lethal doses of lewisite, and to compare the effects of these compounds with those of a monothiol and an alternative treatment with hydrogen peroxide which was under investigation elsewhere at the time of this work.

White rats (80–200 g.) were used. A small area of skin (approx. 10 sq.cm.) on the back of each animal was clipped free from fur with scissors. The animals were not shaved on account of the risk of trauma to the skin. A measured quantity of lewisite, calculated from the Porton figure (LD_{50} dose = 24 mg./kg.) to be between 1 and 2 LD_{50} doses, was then placed on the middle of the shaved area by means of a calibrated capillary pipette. At varying intervals of time after the application of the lewisite a measured amount of BAL was placed on the contaminated site, and was lightly spread over the shaved area with a glass rod, after which the animals were returned to their cages.

In the earlier experiments a large excess of BAL was used in the treatment, approximately 50–70 mg. being applied to a burn caused by 3–8 mg. of lewisite; in Exp. 10 (Table 8), however, the same quantity of BAL was applied to the burn as in the other experiments, but the excess remaining on the skin was rubbed off with cotton-wool at the end of 5 min. In each experiment the animals that survived were kept under observation for at least one month, and in some experiments for 2 months.

These experimental results are summarized in Table 8. The dithiols and the monothiol were all applied in the way described above for BAL; the details of the treatment with hydrogen peroxide are given later.

It will be seen from Table 8 that the local application of BAL is highly effective in saving the lives of rats following contamination with amounts of lewisite that proved uniformly fatal to untreated animals. A survival of 100 % is obtained even when commencement of treatment is delayed for half an hour, after the time of contamination; further, 17 out of 18 animals survived when treatment was delayed for 1 hr., and 14 out of 16 after the elapse of 2 hr.

At the time when this work was reported to the Ministry of Supply, sufficient experiments had not been made with other dithiols to warrant any certain conclusions regarding their particular suitability as therapeutic agents for these lesions.

Table 8. *Summary of inunction treatment of rats contaminated with lethal amounts of lewisite*

Exp.	No. of animals tested	mg. lewisite/ kg. body wt.	Time between contamination and treatment (min.)	Surviving	
				No.	%
1. Untreated	4	27–29	—	0	0
2. „	6	28–33	—	0	0
3. „	6	29–31	—	0	0
4. „	6	31–37	—	0	0
5. „	3	31–34	—	0	0
6. „	4	35–37	—	0	0
1, 2. 2:3-Dimercaptopropanol	8	28–34	5	8	100
2. „	4	27–33	10	4	100
5. „	6	32–36	15	6	100
7, 8, 9. „	17	30–48	30	17	100
10. „	4	34–37	30	4	100
11, 12. „	12	30–36	60	11	92
13. „	6	31–37	60	6	100
6, 14, 15. „	16	29–39	120	14	88
16. 1:3-Dimercaptopropanol	6	32–38	45	4	67
17. Toluene-3:4-dithiol	6	28–35	5	6	100
17. Toluene-3:4-dithiol	6	29–34	15	2	33
18. Propane-1:3-dithiol	5	33–39	60	4	80
18. Ethane-1:2-dithiol	5	34–38	60	1	20
19. 2-Mercaptoethanol	6	30–38	30	0	0
14. Hydrogen peroxide	6	31–38	30	2	33
15. „ „	6	34–38	30	1	17
19. „ „	6	33–39	60	4	67
20. „ „	6	34–39	60	0	0

It may be stated however that the aromatic dithiol, toluene-3:4-dithiol, appeared to be toxic to the rat's skin in the amounts used, a large area of necrosis appearing over the treated area; this has never been seen in the case of the aliphatic dithiols so far investigated.

In the one experiment (19) in which a monothiol was used, all six animals died although treatment was begun 30 min. after contamination.

In order to standardize conditions in the animals treated with hydrogen peroxide each burn was swabbed for exactly 3 min. with pledgets of cotton-wool well soaked in hydrogen peroxide. In all, only 7 out of 24 rats treated in this way survived.

It should be pointed out that in every case marked oedema of the skin and subcutaneous tissues underlying the burn, and in many of the cases diarrhoea (and occasionally lachrymation and salivation) developed within 30 min. of the time of application of the lewisite, so that it would appear that BAL or other suitable dithiols can act not only after the appearance of physical signs at the site of the burn, but also after the development of general signs presumably dependent on the systemic effects of lewisite oxide absorbed from the burn.

In one experiment (13) included in Table 8 the burn was treated at the end of 1 hr. with 10% BAL ointment made up as follows: Hardened palm kernel oil, 8 g.; ethyl phthalate, 8 g.; BAL, 4 g.; talc, 20 g. The ointment was applied in a thin layer over the contaminated site and the surrounding skin. As indicated in Table 8 all six animals so treated survived.

The early treatment of rats following heavy contamination with lewisite was next investigated (Table 9). Groups of animals received applications

Table 9. *Treatment of rats heavily contaminated with lewisite with BAL and hydrogen peroxide*

mg. lewisite/ kg. body wt.	No. of LD_{50} doses applied	No. of animals tested		No. surviving		% surviving	
		BAL	H_2O_2	BAL	H_2O_2	BAL	H_2O_2
173	7	6	6	6	4	100	67
207	9	6	6	6	1	100	17
276	11	6	5	6	0	100	0
345	14	6	12	0	0	0	0

of 7, 9, 11 and 14 LD_{50} doses of lewisite on the clipped skin of the back; 5 min. later they were treated either by the application of BAL to the burn or by swabbing with hydrogen peroxide for 3 min.; in these experiments the excess BAL was not wiped off the contaminated area, but was spread over it with a glass rod.

From the results obtained it appears that BAL can protect completely against the effects of 11 LD_{50} doses of lewisite when treatment is commenced 5 min. after contamination. As in the previous experiments, hydrogen peroxide appears to be less effective than BAL. When 14 LD_{50} doses were applied no survivals were obtained with either form of treatment; this is not altogether surprising since, neglecting the loss of lewisite from the skin by evaporation, in order to protect against such a dose the BAL would have to inactivate well over 90% of the lewisite present, this inactivation commencing 5 min. after the lewisite had begun to penetrate the skin.

It seemed of interest to determine also whether BAL is capable of exerting any prophylactic action when applied to the skin of rats at intervals before the application of lewisite. 100–200 mg. of BAL were therefore spread over the clipped skin in the usual way, and at intervals of from 3 to 23 hr. later measured lethal amounts of lewisite were applied to these areas. It will be seen (Table 10) that in the

Table 10. *Prophylactic action of BAL against lewisite*

Time (hr.)	mg. lewisite/ kg. body wt.	No. of LD_{50} doses applied	No. of animals tested	Survivors	
				No.	%
3	33–38	1·4–1·7	6	6	100
4	34–38	1·5–1·7	6	6	100
8	41–50	1·8–2·2	6	1	17
23	32–37	1·4–1·6	6	5	83

groups of animals tested the inunction of BAL 3 or 4 hr. before the application of $1{\cdot}5 \times LD_{50}$ of lewisite resulted in the survival of all the animals. But since at the end of 4 hr. small amounts of unabsorbed BAL were still present on the surface of the skin this result was not surprising. In a third group of animals therefore $1{\cdot}5 \times LD_{50}$ was applied 23 hr. after inunction with BAL. It was found that even at this interval five out of the six animals survived. The prophylactic action of BAL was therefore investigated against larger doses of lewisite; six animals received $2 \times LD_{50}$ 8 hr. after the application of BAL; of these, only one survived.

From these few experiments it would seem therefore that while BAL may exert considerable prophylactic power over short lengths of time, its effectiveness after longer intervals is questionable.

In contrast to the results obtained with guinea-pigs, to be described later, it should be pointed out that in these rat experiments death from lewisite poisoning, irrespective of whatever form of treatment had been attempted, always occurred within 48 hr., and in the great majority of cases within 24 hr.; late deaths after several days were never seen.

Treatment of lewisite contamination in guinea-pigs

Owing to the possible variations in reaction of different animal species it was considered advisable to investigate the antidotal activity of BAL in guinea-pigs as well as in rats.

Guinea-pigs of 300–500 g. weight were used. As in the rat experiments, an area of skin (approx. 16 sq.cm.) was clipped free of fur with scissors. A lethal dose of lewisite, based on the Porton figure for guinea-pigs (LD_{50} = 11 mg./kg. body wt.) was then placed on the skin, and the burn treated with BAL 1 hr. later. This treatment as in the previous experiments, was again compared with the results obtained by swabbing the burn with hydrogen peroxide for 3 min.; in the two experiments involving hydrogen peroxide, treatment was begun 30 min. and 60 min. after the application of the lewisite respectively. The survivors in each of the two experiments were kept under observation for 1 month.

Table 11. *Comparison of BAL and hydrogen peroxide in guinea-pigs following contamination with lethal amounts of lewisite*

	No. of animals tested	Dose of lewisite mg./kg. body wt.	Time (min.)	Survivors No.	Survivors %
Untreated	4	17–19	—	0	0
BAL	5	19–21	60	4	80
BAL	5	30–35	60	5	100
H_2O_2	5	19–21	30	0	0
H_2O_2	4	18–20	60	0	0
H_2O_2	5	29–34	60	0	0

It will be seen (Table 11) that treatment with hydrogen peroxide, begun 30 or 60 min. after contamination with 2 and $3 \times LD_{50}$ of lewisite, failed to cause the survival of any of the animals. Five guinea-pigs, on the other hand, that had received three lethal doses of lewisite all survived following treatment with BAL 1 hr. after contamination, while four out of five survived following treatment after contamination with $2 \times LD_{50}$ 1 hr. previously.

It was observed in these experiments that although guinea-pigs contaminated with lethal amounts of lewisite did not survive following treatment of the burns with hydrogen peroxide the course of the poisoning was much modified by the treatment; thus, the four untreated animals died under 26 hr.; the five animals treated with hydrogen peroxide after 30 min. died after 10, 12, 12, 15 and 16 days respectively; four animals treated after 60 min. died after 2, 10, 11 and 17 days respectively, while of the five animals that received $3 \times LD_{50}$ of lewisite followed by peroxide treatment after 60 min. two died on the first day and the remaining three on the fifth day. Moreover, apart from the fact that the animals were not eating well and were steadily falling in weight, they did not appear to be seriously ill until one or two days before death occurred. At autopsy, however, those that died late showed very marked pathological changes in the liver and gall-bladder accompanied by extensive peritoneal effusion. A possible explanation of the chronicity of the poisoning resulting from treatment with hydrogen peroxide may be that the lewisite in the skin is first oxidized to the less toxic pentavalent form; this subsequently undergoes a gradual reduction leading to a slow liberation of the toxic trivalent form into the circulation. The one death among the animals treated with BAL took place on the 13th day, after a steady fall in body weight. The remaining animals treated in this way showed only a very slight and transient fall in weight, and when they were killed one month after commencing the experiment they were eating well and were all in apparently good condition.

Antidotal activity of BAL administered by injection

In view of the likely possibility of systemic poisoning ensuing from the absorption of arsenic from skin heavily contaminated with lewisite, it was clearly important to determine whether a dithiol given by injection, can prevent death from arsenical intoxication. Although for various reasons BAL is not an ideal compound for injection, it is soluble in water and neutral salt solutions to the extent of about 6 g./100 ml.

Groups of white rats were injected intramuscularly with lethal amounts of neutralized solutions of sodium arsenite. Considerable individual variations in sensitivity were observed among different animals; moreover, if death did not take place within a few hours of injection, the animals recovered and survived; in only one case did a late death (after 4 days) occur. Groups of rats that had received similar doses were treated with BAL after varying intervals of time. The BAL (0·1–0·3 ml. saturated aqueous solution) was given by intraperitoneal injection in order to exclude the possibility of any local detoxication at or near the injection site; most of the animals were seriously sick or even collapsed at the time of the injection. The results of these experiments are shown in Table 12.

Two experiments towards the same end were next carried out in rats that had received approximately $1{\cdot}5 \times LD_{50}$ of lewisite on the clipped skin of the back. In the first experiment two groups of rats were each given subcutaneous injection of a saturated aqueous solution of BAL 1 and 2 hr. after contamination respectively; in the second experiment both groups of animals received a second small dose of BAL 3½ hr. after the first. The results are given in Table 13.

Table 12. *Antidotal activity of injected BAL in acute poisoning with sodium arsenite*

	No. of animals	Dose of poison (mg. As_2O_3/kg.)	Interval (min.) between poisoning and treatment	Survivors No.	Survivors %
Untreated	7	8·5–9·9	—	5	71
	15	10·0–14·6	—	5	33
	6	15·0–17·3	—	0	0
	6	18·1–22·0	—	0	0
Treated	10	14·0–18·0	20	9	90
	6	17·4–18·8	12	6	100
	12	18·1–22·4	7	8	67

Table 13. *Effect of BAL given by subcutaneous injection after lewisite contamination of the skin*

	No. of animals	mg. lewisite/kg.	Survivors No.	Survivors %
(1) Dose: 50–61 mg. BAL/kg.				
BAL after 1 hr.	6	34–38	4	67
BAL after 2 hr.	6	32–38	5	83
(2) Dose: 60–70 mg. BAL/kg., followed after 3½ hr. by a further 30–35 mg./kg.				
1st dose after 1 hr.	6	33–38	6	100
1st dose after 2 hr.	6	32–39	6	100

Human tests

Human tests were first carried out in Oxford on four volunteer subjects. The skin of the forearm was contaminated with measured amounts of undiluted lewisite; after intervals of up to half an hour after contamination the burns were treated with BAL, the results being compared with untreated lesions, or, in the case of one subject, with a lesion treated with 2-mercaptoethanol.

In the cases treated with BAL, the compound (30 mg.) was applied to the contaminated site and then lightly spread over a small surrounding area; after a few minutes the BAL was gently rubbed into the skin. On two of the subjects (A.G.O. and R.B.F.) a drop of olive oil was placed on the 'untreated' control burn and gently rubbed in, in order to control any possible spreading effect on the lewisite resulting from the rubbing of the skin. In one subject (V.P.W.) the two contaminations were made on corresponding spots on the two forearms; in the other cases all the contaminations on each subject were made on the same arm, the untreated control being placed between the two treated burns. The results of these tests are given in Table 14, the readings being taken 24 hr. after contamination. It will be seen that the local application of BAL produces a striking effect on the course of the

Table 14. *BAL treatment of lewisite burns in man*

Subject	mg. lewisite applied	Untreated	BAL after 5 min.	BAL after 15 min.	BAL after 30 min.	2-Mercaptoethanol after 30 min.
R.H.S.T.	0·5	V (1 × 0·3)	Nil	—	—	—
R.H.S.T.	0·5	V (1 × 0·6)	—	Nil	E (0·6 × 0·4)	—
A.G.O.	0·5	V (0·7 × 0·7)	—	S (0·2 × 0·2)	E + (1 × 0·7)	—
R.B.F.	0·5	V (1 × 0·5)	—	E + (0·5 × 1)	E − (0·9 × 0·3)	—
V.P.W.	1·0	—	—	—	Nil	E + (1·2 × 1·2) V (0·9 × 0·7)

S = oedema, no erythema. E − = faint erythema. E = erythema. E + = erythema and oedema. V = vesication.
(Dimensions recorded in cm.)

Table 15. *Human tests carried out in the Physiological Department, Porton*

Subject no.	Contamination	Duration of contamination (min.)	Treatment: BAL	Treatment: H_2O_2	Treatment: Olive oil
			Exp. 1		
1	1·1 mm. drop lewisite	10	Very small E −	Very small V	—
2	,, ,, ,,	15	Very small E −	V (1·8 × 1·0)	—
3	,, ,, ,,	30	Very small E −	E + (1·3 × 1·2) Central V	—
4	,, ,, ,,	15	Nil	—	V (1·8 × 0·8)
			Exp. 2		
1	1·1 mm. drop lewisite	45	E − (1·6 × 1·6)	V (2·2 × 1·1)	—
2	,, ,, ,,	45	Small E −	V (1·3 × 0·7)	—
3	,, ,, ,,	60	Nil	Small E −	—
4	,, ,, ,,	60	Nil	Pinhead V E + (2·0 × 1·5)	—
5	2 mm. drop lewisite	30	E (2·2 × 1·2)	V (3·0 × 1·3)	—
6	,, ,, ,,	30	E (1·9 × 1·3)	E + (2·7 × 2·5) V (2·5 × 2·3)	—

(Symbols as in Table 14.)

lesion, no vesication occurring in any of the four subjects even when treatment was not begun till 30 min. after contamination. Treatment with 2-mercaptoethanol did not prevent the development of a large, well filled vesicle.

Further tests on human volunteers were carried out at Porton by arrangement with the Superintendent of the Physiological Department. The results of these tests are shown in Table 15 for comparison with the tests performed in Oxford. Here again, BAL was compared with olive oil and also with hydrogen peroxide. In these experiments the BAL was lightly rubbed over the contaminated area with a glass rod for 3 min., then with the finger at intervals up to 5 min., when the excess was wiped off; in Exp. 1 hydrogen peroxide was swabbed on the comparison lesions for 30 sec., while in Exp. 2 after swabbing for 30 sec. a cotton-wool swab saturated with hydrogen peroxide was placed over the area for 5 min. and kept well saturated during this period. Both BAL and hydrogen peroxide were also tested alone for intrinsic irritancy to normal skin, but in neither case was there any evidence of irritation at the 24 hr. reading.

Further human tests have since been carried out at Porton against phenyldichloroarsine; it has been found that BAL can also prevent vesication by this arsenical up to intervals of 1 hr. after contamination.

Toxicity of dithiols

When this work was being carried out no pharmacological information was available concerning the actions or toxic effects of dithiols in the body. The lethal dose of BAL to rats was first established by application to the skin and by intraperitoneal injection. In the cutaneous applications the BAL was spread over the clipped backs of rats; the amounts that were necessary to reach the lethal dose were so great that they remained as a thick film of oil, on the surface of the skin, complete absorption not taking place for some hours. A proportion of the amount applied was therefore lost, being rubbed off on to the fur surrounding the clipped skin; further, owing to the animals licking the anointed skin and surrounding fur, small quantities of BAL must also have been swallowed. It follows therefore that the lethal dose by cutaneous application is only an approximate figure, although in good agreement with that subsequently found independently at Porton.

For intraperitoneal injection the BAL was dissolved in freshly distilled thiodiglycol (10 mg./0·1 ml. thiodiglycol). The results of administration of BAL by these routes are shown in Table 16; very roughly, 2–3 g. BAL/kg. body weight are lethal when applied to the skin of rats, and 150 mg./kg. when injected intraperitoneally.

Table 16. *Toxicity of BAL to rats*

Mode of administration	No. animals tested	mg. BAL/kg.	Survivors No.	Survivors %
Cutaneous	11	1000–2000	11	100
	11	2030–3000	6	55
	4	4400–6350	0	0
Intraperitoneal	4	86–98	4	100
	6	100–113	3	50
	10	124–145	4	40
	8	147–167	0	0

A more accurate determination of the LD_{50} dose of a standard preparation of water-purified BAL given by intramuscular injection into white rats was later undertaken. Porton strain white rats, weighing from 120 to 180 g. were used, the animals being starved 24 hr. prior to the test. The BAL was dissolved in an amount of freshly distilled propylene glycol so as to give the required dose in a total volume of 0·1 ml. The injections were made into the hind leg, using a tuberculin syringe. Fifteen rats were used for each dosage level. The animals within any one group were chosen of such weights that in any group the dose (as mg./kg. body weight) never varied by more than ± 5 mg./kg. from the level stated in Table 17. The number of rats dying within

Table 17. *Toxicity to white rats of standard preparation of BAL (intramuscular injection)*

(15 rats in each group)

Dose (mg./kg.)	No. rats dead	% dead
95	1	6·5
105	6	40
115	9	60
125	12	80
135	14	93
175	15	100
205	15	100

72 hr. (in our experience it has been most exceptional for any deaths to occur after this time), together with a summary of the statistical examination of the result (kindly carried out by Dr R. B. Fisher, of this Department) are shown in Table 17. The LD_{50} dose, estimated by Bliss's method, is 113 mg./kg.; more precisely, $\log_{10}(LD_{50}) = 2{\cdot}0527 \pm 0{\cdot}0112$ (the slope of the line connecting log dose (mg./kg.) and probit is $19{\cdot}610 \pm 1{\cdot}353$). This corresponds to a probability of approximately 1 chance in 5 that log (LD_{50}) will be outside the range $2{\cdot}0527 \pm 0{\cdot}0172$, i.e. there is 1 chance in 10 of obtaining a value of LD_{50} below 108·5 or above 117·5 mg./kg. body weight. Therefore the LD_{50} dose = 113 mg./kg. $\pm$ 2·6 %. It must be stressed that this estimate applies only to the conditions used here and to this particular strain of rats.

A few rough toxicity determinations of other related dithiols were also made. In all cases except where stated the thiol was dissolved in thiodiglycol, and 0·2 ml. of the solution injected intramuscularly

Table 18. *Toxicities of other dithiols (by intramuscular injection into rats except where stated)*

Compound	No. rats tested	Dose (mg./kg.)	Survivors No.	Survivors %
1:3-Dimercaptopropanol	6[1]	35– 67	0	0
	6	26– 32	2	33
	6	17– 20	4	67
Propane-1:2-dithiol	6	90–112	0	0
	5	48– 53	1	20
	6	25– 33	4	67
Propane-1:3-dithiol	6	94–103	0	0
	6	43– 54	6	100
Pentane-1:5-dithiol	6	77– 90	5	83
	6	45– 48	6	100
2:3-Dimercaptopropylamine hydrochloride[2]	6	77– 89	0	0
	6	36– 38	0	0
	6	20– 23	5	83
Trimercaptopropane	6	76– 85	1	17
	6	24– 25	6	100

[1] By intraperitoneal injection into these 6 rats.
[2] Dissolved in water and adjusted to pH 6·6–7·0.

into the hind leg. Table 18 summarizes these determinations.

It is of interest that the LD_{50} dose of 1:3-dimercaptopropanol, reckoned from the figures given in Table 18, is of the order of only 20–30 mg./kg., so that this compound is about 5 or 6 times as toxic as the 2:3-isomer.

None of the other compounds examined has been found to be less toxic than BAL. In all experiments except those with propane-1:2-dithiol, death took place within 48 hr., and usually within 24 hr. Lethal amounts of these dithiols cause tremors leading to death in convulsions, severe spasm of the muscles of the abdominal wall being noticed shortly before death. Propane-1:2-dithiol is the exception; lethal amounts of this compound cause the animals to pass into a collapsed condition with a profound fall in body temperature (down to 23–29°), the animals remaining in this condition for many hours before death (up to 48 hr. or longer).

Slightly sublethal amounts of BAL will also give rise to convulsions, occurring 1–2 hr. after injection. With smaller amounts still, of the order of $\frac{1}{3}$–$\frac{1}{2}$ lethal dose, it is possible to give repeated doses to rats at 3–4 hr. intervals without the development of any serious or lasting symptoms.

In two experiments, 0·5 and 1·0 ml. respectively of undiluted BAL were applied to the forearm of a volunteer. 0·5 ml. was sufficient to cover the whole of the volar aspect of the forearm, the total area covered being about 300 sq.cm.; in the second experiment 1·0 ml. was spread over the entire forearm from wrist to elbow, the area covered being about 530 sq.cm. A rash, scarlatiniform and slightly oedematous in type, and accompanied by severe tingling, developed rapidly, but disappeared completely within 4 or 5 hr. Slight lachrymation and dilatation of subconjuctival vessels were also observed, but it was impossible to decide whether this was due to the effects of absorbed BAL or to the direct action of the vapour.

In the course of the therapeutic tests carried out on human subjects both in Oxford and Porton, tingling and transient slight erythema were produced in most subjects by the application of the undiluted compound, and in a few cases a localized urticarial response centring round the hair-follicles was observed. These signs of irritancy, however, were all of short duration, usually disappearing in under 4–6 hr.

Toxicity of the lewisite derivatives of thiols

A comparison has been made of the toxicity to rats (by subcutaneous injection) of the lewisite derivatives of 2:3-dimercaptopropanol and of 2-mercaptoethanol.

The lewisite-BAL derivative was prepared as described by Stocken (1946). Samples of the compound were dissolved in thiodiglycol and injected subcutaneously into rats; the LD_{50} dose of lewisite by subcutaneous injection in rats is 5 mg./kg. Table 19 gives the results obtained with the lewisite-BAL compound. The figures in column 3 of Table 19 give the number of LD_{50} doses of

Table 19. *Toxicity of the lewisite derivative of BAL*

No. rats tested	mg./kg. body wt.	Equivalent no. of LD_{50} doses of lewisite	Survivors No.	Survivors %
4	12·5–18·1	2–3	4	100
4	21·0–23·4	3–4	4	100
14	25·6–30·4	4–5	7	50
3	40·5–58	6–9	0	0

lewisite contained in the amount of the derivative injected. It will be seen that on the basis of the arsenic content the LD_{50} dose of this compound is about 5 times that of lewisite, indicating that combination of lewisite with BAL to form the dithiarsenole ring brings about a very substantial decrease in toxicity.

We have not so far been able to obtain a pure specimen of the lewisite derivative of 2-mercaptoethanol; from the analytical figures it was calculated that the product contained approximately 11 % of free lewisite oxide. An amount of 2-mercaptoethanol equivalent to the free lewisite oxide was therefore added to the product, and a sample of this mixture, dissolved in thiodiglycol, was injected subcutaneously into rats. The dose of the lewisite compound together with the free lewisite oxide present was equivalent to $1{\cdot}88$–$2{\cdot}06 \times LD_{50}$ doses of lewisite; the free lewisite oxide present, which was

'neutralized' by the added 2-mercaptoethanol, corresponded to less than one-fifth of an LD_{50} dose. The result of this experiment is shown in Table 20.

Table 20. *Toxicity of the lewisite derivative of 2-mercaptoethanol*

No. of rats tested	mg./kg. body wt.	Equivalent no. of LD_{50} doses of lewisite	Survivors No.	Survivors %
6	13·0–14·6	1·8–2·1	0	0

It will be seen that all six rats died following the injection of an amount of the derivative equivalent to 2 LD_{50} doses of lewisite; in the case of the lewisite-BAL derivative 100% survival was obtained following the injection of an amount equivalent to 3–4 LD_{50} doses.

DISCUSSION

The results presented above indicate clearly that, by contrast with the monothiols so far investigated, a simple aliphatic dithiol, 2:3-dimercaptopropanol (BAL), is capable of exerting a marked antagonistic effect to the toxic action of lewisite on animal cells.

In testing these thiols the results obtained with the brain pyruvate oxidase system, which throughout the work has been used as a rapid and economical initial test system, have been amply confirmed by *in vivo* tests on rats, guinea-pigs and human subjects.

The local treatment of lewisite burns of the skin in man has been tested up to 60 min. after contamination, and the results show that BAL is a highly effective agent in the prevention of vesication. In most of the experiments the BAL was not applied to the lesion until well-marked signs of injury to the skin (erythema and oedema) had developed, and it is of particular interest that successful therapy can be obtained when delayed until this late stage. Since in many of the cases the final erythema 24 hr. after treatment was considerably less in size and intensity than that already developing at the time of treatment, an actual reversal of the underlying pathological change brought about by the lewisite is suggested. Furthermore, the ability of BAL to reverse the poisoning of the pyruvate oxidase system by lewisite oxide indicates that a similar reversal of the combination of lewisite with the arsenic 'acceptor' in living skin is certainly possible.

The systemic effects and ultimate deaths of animals contaminated on the skin by lethal amounts of lewisite can also be prevented by the local application of BAL to the lesion. In these experiments the treatment with BAL was successful when begun after the appearance of physical signs of intoxication (diarrhoea, lachrymation and salivation), presumably dependent on the systemic effects of lewisite oxide absorbed from the damaged skin. BAL, given by injection, has also been found to save animals after either a lethal contamination with lewisite or the injection of a rapidly fatal dose of sodium arsenite, indicating that BAL is not only an effective skin decontaminant, but can also deal with systemic poisoning.

Since no late deaths in either rats or guinea-pigs have been seen following the treatment of lethal lewisite contaminations with BAL it seems unlikely that the lewisite-BAL compound dissociates inside the body to any great extent, with reliberation of a toxic arsenical compound. *In vitro* confirmation of this view is afforded by the enzyme experiments in which a mixture of equivalent amounts of lewisite and BAL proved almost non-toxic to the pyruvate oxidase system and showed no increase in toxicity throughout the 2 hr. of the experiment. As later work has shown (Thompson & Stocken, 1941), the arsenic is in fact rapidly excreted from the system in increased amounts following treatment with BAL.

A brief, preliminary review of the English work on British Anti-Lewisite has already appeared (Peters, Stocken & Thompson, 1945).

SUMMARY

1. The behaviour of monothiols and dithiols in their reactions with lewisite and arsenite has been examined by comparing the abilities of representative compounds of these classes to protect the brain pyruvate oxidase system, to cause survival of animals contaminated with lewisite or injected with arsenite, and to prevent vesication in man.

2. The relative rates of hydrolysis of thioarsinites prepared by reacting lewisite with mono- and dithiols have been compared; the cyclic thioarsinites formed with dithiols are markedly more stable than the non-cyclic compounds formed with monothiols.

3. Low concentrations of 2:3-dimercaptopropanol (BAL) are highly effective in protecting the brain pyruvate oxidase system from the toxic action of chlorovinyl-, phenyl-, ethyl- and methyl-arsenoxides and of sodium arsenite. Other 1:2- and 1:3-dithiols have also been tested and found effective against chlorovinylarsenoxide.

4. A mixture of lewisite oxide and BAL in equivalent proportions, is almost non-toxic to the pyruvate system, when tested at a concentration of lewisite which alone would produce about 50% inhibition.

5. BAL is capable of bringing about a substantial degree of reversal of the lewisite inhibition of the pyruvate system.

6. BAL prevents the inhibition of the respiration of rat skin slices by lewisite; 2-mercaptoethanol and cysteine were ineffective.

7. Survival of rats and guinea-pigs after contamination of the skin with lethal amounts of lewisite has been obtained by the local application of BAL even when treatment was delayed for as long as 2 hr. after contamination.

8. Injections of saturated aqueous solutions of BAL have saved rats after the injection of lethal amounts of sodium arsenite or the contamination of the skin with lethal amounts of lewisite.

9. Vesication in man following the application of small amounts of lewisite to the skin can be prevented by treatment with BAL up to at least 1 hr. after contamination.

10. Rough toxicity tests of a number of dithiols have been carried out and the LD_{50} dose of BAL when given by intramuscular injection to white rats has been accurately assessed.

This work was carried out for the Ministry of Supply as part of a larger programme under the direction of Prof. R. A. Peters, M.C., F.R.S. Our acknowledgements are due to Surg.-Capt. A. Fairley, R.N., for facilities provided in the Physiological Department, Experimental Station, Porton, and for his co-operation and advice in carrying out the later human tests. We also wish to thank the Chief Scientific Officer, Ministry of Supply, for permission to publish this work, Dr V. P. Whittaker for his help in some of the preparations, and Miss M. R. Kempson and Messrs C. Dear and W. Weinstein for their skilled assistance.

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British Anti-Lewisite

3. ARSENIC AND THIOL EXCRETION IN ANIMALS AFTER TREATMENT OF LEWISITE BURNS

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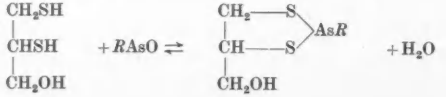
(*Received* 5 *April* 1946)

Earlier work in this laboratory (Stocken & Thompson, 1941) has shown that 2:3-dimercaptopropanol (British Anti-lewisite or BAL) reacts with lewisite and other arsenicals of the types *R*AsO and *R*AsCl$_2$ to form stable ring compounds:



the affinity of the dithiol for the arsenical being sufficiently great to bring about combination with the traces of lewisite dissociating from combination with the tissue proteins. Low concentrations of BAL have been shown to protect against and to reverse the toxic action of lewisite on the pyruvate oxidase system of brain, in this respect differing fundamentally from all the monothiols so far investigated. Further, small animals may be caused to survive after contamination with lethal

amounts of lewisite by the application of BAL to the burn as late as 1 hr. after contamination; and finally, vesication by lewisite or phenyldichloroarsine in man may be prevented by treatment with BAL after intervals up to 1 hr. following contamination.

In view of these results it is clearly desirable to know something of the metabolism and ultimate fate of lewisite absorbed from the skin, and of any changes in its metabolism or rate of excretion brought about by treatment of the contaminated skin with BAL.

The excretion of arsenic, and in particular of the organic therapeutic arsenicals, has been extensively studied in animals and man (Heiden & Navassart, 1913; Voegtlin & Thompson, 1922; Underhill & Davis, 1922; Bulmer, 1923). It is now known that arsenic appears in the urine within a few hours of its intravenous injection. In rats, trivalent arsenoxides show the slowest rate of excretion; arsenobenzene derivatives (i.e. of the type *R*As : As*R*) a much greater excretion rate, and pentavalent compounds greater still. The maximum excretion rate occurs on the day of the injection or on the following day, but small amounts continue to be excreted for a large number of days. In the case of certain compounds, notably arsphenamine, a considerable quantity is excreted, via the biliary tract, in the faeces.

Calder (1941) studied the excretion of arsenic after the application of lewisite to the skin of rabbits, and found that 3–6% of the amount applied was excreted in the urine during the first day after contamination, with a further 6–7% during the second day. In all, he found that 15–20% of the amount applied was excreted by the kidneys during the first week, but that by the end of this time no further excretion was taking place. Arsenic was also present in the bile during the first few days, but all tests carried out on faeces were negative.

The influence of thiols in this connexion does not appear to have been studied, although an extensive literature exists on the effect of sodium thiosulphate on arsenic excretion. This compound was used by Ravaut in 1920 for the treatment of arsphenamine dermatitis; since then, much conflicting evidence has been produced both for and against the value of this compound in the treatment of various conditions due to both acute and chronic arsenical intoxication. In 1925 Myers, Groehl & Metz claimed that patients with arsphenamine dermatitis or jaundice excrete a large amount of arsenic in the urine after each injection of sodium thiosulphate, and they attributed the beneficial effect in part to the removal of stored arsenic from the body. Kuhn & Reese (1925) put forward similar claims, while Ayres & Anderson (1938) observed that an increase in the urinary excretion of arsenic usually follows the injection of sodium thiosulphate. Other workers, however, have been unable to confirm this increased excretion of arsenic, and have, in some cases, found an actual decrease (Mattice & Weisman, 1937; Mattice, Baxt & Byrne, 1940).

Experiments are described below which were designed to compare the rates of urinary and faecal excretion of arsenic from the body after lewisite contamination in untreated animals and in animals which had received treatment with BAL. Further, since visible damage to the skin may be largely or completely prevented by treatment of the contaminated site with BAL after an interval sufficient to allow penetration of the skin by the lewisite, estimations were carried out to determine the effect produced by BAL on the amount of arsenic left in the skin 48 hr. after contamination.

Since, at the time when this work was first reported (Thompson & Stocken, 1941), no information was available concerning the biochemical effects or metabolism of dithiols in the animal body, preliminary experiments on the metabolism of BAL are also presented.

EXPERIMENTAL AND RESULTS

Excretion of arsenic after cutaneous application of lewisite to rats, and the effects of BAL treatment

Twenty-four hr. specimens of urine and faeces were obtained from rats following the cutaneous application of small amounts of lewisite, and the total quantity of arsenic excreted in these periods determined.

Adult white rats weighing from 150 to 350 g. were used throughout these experiments. A small area of skin (approx. 9 sq.cm.) was clipped free from fur with scissors. A dose of lewisite was then placed on the middle of the clipped area, and the animals either returned directly to metabolism cages, or treated with BAL after measured intervals and then placed in the cages. The BAL was dropped on to the contaminated area from a pipette and lightly spread over the clipped skin with a glass rod.

Owing to the small quantities of lewisite used in these experiments it was found necessary to place two or three rats in each metabolism cage, and to estimate the arsenic in the pooled excreta. This pooling had the additional advantage of diminishing the effects of the individual variations in the rate of excretion of arsenic that are known to exist among animals of the same species (Voegtlin & Thompson, 1922).

Arsenic was estimated by a modification of the titrimetric method of Allcroft & Green (1935), 97–100% recovery of amounts of arsenic varying from 50 to 300 μg. being obtained with this method. The urine and faeces were each digested with a perchloric-nitric acid mixture, 93% recovery of added arsenic being obtained from urine using this digestion method, and 99% from faeces. All estimations were done in duplicate.

Table 1 shows the effect of BAL treatment on the excretion of arsenic in the urine of lewisite-contaminated rats. The untreated control rats were

contaminated with amounts equivalent to 10·0–12·7 mg./kg. body weight (i.e. approximately 0·5 × LD_{50} dose); in the first 24 hr. the arsenic excreted in the urine corresponded to 2–4 % of the amount of lewisite applied to the skin.

The treated animals received an application of BAL 15 min. after contamination; in no case was any lewisite to be seen on the skin at the time of treatment. The urinary excretion of arsenic in the from the untreated cages was in a few cases contaminated with small amounts of fluid or semi-fluid faecal matter, so that the true values obtained for the urinary excretion from the untreated animals may be slightly lower than those actually determined; estimations of the faecal excretion of arsenic, to be described later, indicate however, that the error arising from slight faecal contamination of the urine specimens would be insignificant.

Table 1. *Urinary excretion of arsenic after cutaneous application of lewisite to rats*

mg. lewisite/kg. body wt.	Total amount As applied (mg.)	Arsenic recovered in urine: mg./24 hr. specimen 0–24 hr.	mg./24 hr. specimen 24–48 hr.	Percentage of amount applied 0–24 hr.	Percentage of amount applied 24–48 hr.
(1) Untreated					
11·7–12·7	4·54	0·12	0·07	2·5	1·5
10·0–10·8	2·82	0·10	—	3·5	—
10·3–10·7	3·38	0·07	0·05	2	1·5
10·2–10·9	3·31	0·14	—	4	—
10·0–10·4	2·70	0·08	0·08	3	3
(2) BAL treated (after 15 min.)					
49·5–57·0	5·40	0·78	—	14·5	—
49·2–50·8	6·49	1·55	—	24	—
45·5–50·0	4·87	0·69	0·17	14	3·5
17·4–19·6	2·16	0·44	0·08	20	4
12·9–15·0	4·54	0·50	0·10	11	2
10·9–13·1	3·49	0·38	—	11	—
10·8–11·8	3·75	0·60	—	16	—
10·0–10·4	2·27	0·29	0·10	13	4·5
(3) BAL treated (after 1 hr.)					
11·9–12·5	2·16	0·72	0·05	33·5	2·5
11·1–12·5	2·16	0·58	0·06	27	3

first 24 hr. in this group corresponded to 11–24 % of the amount applied. It will be seen (Table 1) that treatment after 15 min. caused a marked increase (approximately fourfold) in the urinary excretion of arsenic even when the same contamination (in mg. lewisite/kg. body weight) was used in the two groups of animals. Heavier contamination of the untreated rats was impossible owing to one or more of the animals dying within the first 24 hr. In the treated animals, however, contamination with more than 2 LD_{50} was carried out, the treatment resulting in the excretion of amounts of arsenic corresponding to 14–24 % of the amount of lewisite applied.

In a few experiments the urine was also collected throughout the second 24 hr. period after contamination, but the amount of arsenic excreted in the urine of the treated animals had fallen, although the few experiments that have been done suggest that even in the second 24 hr. excretion may be slightly higher in the treated group.

In contrast to the treated animals all the untreated rats showed varying degrees of diarrhoea, and in some cases visible amounts of mucus were passed with the faeces. For this reason the urine

Two experiments are also included in Table 1 in which BAL treatment was not begun until 1 hr. after contamination, as it was felt that although no liquid lewisite was visible on the skin after 15 min., treatment begun then might not have allowed sufficient time for the lewisite to have penetrated deeply into the skin, so that the application of BAL might have prevented the evaporation of further small amounts of lewisite from the superficial layers of the skin after 15 min. In these two experiments the urinary excretion in the first 24 hr. was even higher than in the animals treated after 15 min., amounting to 33·5 and 27 % respectively of the amount of arsenic applied to the skin. This higher excretion may be due merely to individual variations, but it is clear that treatment after an interval as long as 1 hr. also brings about a very significant increase in the renal elimination of arsenic from the system.

Large doses of BAL administered subcutaneously to rats have a diuretic effect, so that it might be argued that part of the increased excretion of arsenic in the treated animals is due to an increased urine flow; the urine volumes obtained in the above

experiments varied widely, but there was no obvious difference between the two groups of animals, so that BAL applied to the skin in the amounts used in these experiments does not appear to exert any significant diuretic action. Further, Underhill & Davis (1922) have shown that there is no relation between the amount of arsenic excreted and the volume of urine passed after administration of arsphenamine or neo-arsphenamine.

In three experiments the faecal excretion of arsenic was also determined (Table 2); as BAL treatment did not significantly affect the small excretion of arsenic in the faeces, no further faecal analyses were made.

Table 2. *Faecal excretion of arsenic after cutaneous application of lewisite*

	Total amount As applied (mg.)	Arsenic recovered in the faeces: mg./24 hr. faeces 0–24 hr.	mg./24 hr. faeces 24–48 hr.	Percentage of amount applied 0–24 hr.	Percentage of amount applied 24–48 hr.
Untreated	3·38	0·10	0·15	3	4·5
,,	2·70	0·03	0·05	1	2
BAL-treated	5·40	0·04	—	0·75	—
,,	2·27	0·07	0·07	3	3

One further experiment was carried out to study the excretion of arsenic after the subcutaneous injection of the lewisite-BAL compound (Stocken, 1946) into animals. Three rats, weighing 95, 97 and 106 g. respectively, each received a subcutaneous injection of 2·48 mg. lewisite-BAL compound dissolved in 0·1 ml. thiodiglycol. This amount is equivalent to 2 mg. lewisite, so that each rat received an amount of the compound, the lewisite equivalent of which was equal to 4 LD_{50} doses (based on the Porton figure for the LD_{50} dose for lewisite, viz. 5 mg./kg. body weight when administered subcutaneously in rats).

The rats were placed in one metabolism cage, and the pooled urine collected over the next three 24 hr. periods, and analyzed for arsenic. In the first two 24 hr. periods 0·504 and 0·042 mg. As respectively were excreted by the three rats. On the third day no arsenic could be detected in the urine. Since in all, 7·44 mg. lewisite-BAL compound (equivalent to 2·15 mg. As) had been injected into the animals, the recovery of 0·546 mg. in the first 48 hr. corresponds to a urinary excretion of 25 % of the arsenic applied.

Since lewisite vesication in man may be prevented by the application of BAL after erythema and oedema of the skin have already developed, it is evident that up to this stage of damage to the skin cells, BAL can reverse the toxic action of lewisite. It is of interest therefore to determine whether BAL causes any diminution in the amount of arsenic persisting in the skin. In untreated animals the visibly damaged skin, together with a surrounding ring of skin of normal appearance about 0·5 cm. in width, was therefore excised from the killed animal 48 hr. after contamination. In the case of the BAL-treated rats, visible trauma to the skin was in all cases much less, in some animals only slight puckering of the skin marking the site of contamination. From these animals an area of skin approximately equal in size to the pieces removed from the untreated animals was excised. The scars from the three (or two) animals in any experiment were digested together, and the arsenic estimated in the digest as in the excretion experiments, 94 % recovery of added lewisite to normal skin being obtained by this method. The results of these experiments are shown in Table 3.

With small doses of lewisite (9·4–12·5 mg./kg. body weight), the actual amount of arsenic left in the skin of the untreated animals 48 hr. after contamination is in all cases greater than in the treated animals. In one experiment (no. 354) the treated animals received 45–50 mg. lewisite/kg. body weight, yet even with this large dose the amount of arsenic

Table 3. *Effect of BAL treatment on the amount of arsenic remaining in the scar after 48 hr.*

Exp.	No. of rats	mg. lewisite/kg. body wt.	As applied (mg.)	As found (mg.)	Percentage of amount applied
450 Untreated	3	10·3–10·7	3·38	0·24	7·1
452 ,,	3	10·0–10·4	2·70	0·12	4·4
456 ,,	2	9·4–10·6	1·73	0·18	10·4
463 ,,	3	10·4–10·9	1·73	0·14	8·1
464 ,,	2	10·7–10·8	1·30	0·13	10·0
354 BAL-treated	3	45·5–50·0	4·87	0·12	2·5
452 ,,	3	10·0–10·4	2·27	0·06	2·6
456 ,,	3	10·8–11·2	2·27	0·04	1·8
461 ,,	3	11·9–12·5	2·16	0·09	4·2
462 ,,	3	11·1–12·5	2·16	0·045	2·1

present in the skin after 48 hr. was less than the amount present in four out of five of the untreated groups that had received only one-fifth of this dose of lewisite. In Exps. 354, 452 and 456 BAL treatment was begun 15 min. after contamination, in Exps. 461 and 462 1 hr. after contamination. Expressed as percentages of the amounts of arsenic applied to the skin (as lewisite) the difference between the two groups of animals becomes even more striking.

The metabolism of BAL

In the early treatment of lewisite contamination of the skin with BAL, present experience suggests that one application of the drug should suffice, so that the toxic action of large amounts of the compound should not be a complicating factor except in those cases where heavy contamination with lethal amounts of lewisite has occurred. It is possible however that BAL, or some related dithiol, may have an application in the late treatment of lewisite casualties at a time when symptoms of systemic arsenical poisoning are developing; in such cases it might be desirable to keep a low concentration of the drug in the blood for some time, along the lines of therapy with members of the sulphonamide group. In this connexion it was desirable to gain information concerning the length of time that BAL will persist inside the body.

An attempt was therefore made to follow the rate of excretion of BAL, or of a derivative of BAL or some compensatory thiol in the urine by iodine titration. Using rats, the normal daily excretion of thiols and of non-thiol substances titrating with iodine was found to fall within sufficiently narrow limits for the purpose of these experiments.

The procedure adopted was to place the rats in metabolism cages and to follow the excretion of thiols over a period of a few days, the 24 hr. urine samples being separated from faeces and food residues by means of glass separators. After establishing the normal thiol excretion rate, sublethal doses of BAL dissolved in thiodiglycol were then administered to the rats by subcutaneous injection, and the animals returned to the cages for the collection of further urine samples. The thiol content of the 24 hr. specimens of urine was estimated by titration with 0·02 N or 0·01 N-iodine. In order to obtain a satisfactory end-point it was found necessary to carry out the titration in strongly acid solution; 1 ml. of 25% (w/v) trichloroacetic acid was therefore added to each 5 ml. sample of urine. In the later experiments, 1 ml. of N-HCl was placed in the urine separator before beginning to collect each sample, in the hope of diminishing the loss of thiol by oxidation by air during the 24 hr. period; this was found however to have little effect on the amount of titratable thiol present.

The result of a typical experiment is shown in Table 4. It will be seen from these results that there is a marked rise in the iodine titre of the 24 hr. urine specimen following injection of BAL over the mean pre-injection level. Expressed in terms of BAL, the increases in the various experiments

Table 4. *Urinary excretion of thiols after injection of BAL into rats*

(Three rats, weighing 76, 81 and 112 g. resp.; 24 hr. specimens of urine collected for 22 days; 10 mg. BAL dissolved in thiodiglycol injected subcutaneously into each after 4, 14 and 17 days.)

	Days	Vol. 24 hr. urine (ml.)	Vol. 0·02 N-iodine/24 hr. specimen (ml.)	
	1	17·0	0·54	
	2	11·8	0·28	Mean = 0·29
	3	10·6	0·17	
	4	9·4	0·15	
BAL injected				
	5	19·2	**4·76**	
	6	15·0	0·56	
	7	5·4	0·28	
	8	12·5	0·81	
	9	12·0	0·48	
	10	9·6	0·29	Mean = 0·43
	11	12·8	0·44	
	12	10·5	0·46	
	13	12·0	0·36	
	14	7·0	0·20	
BAL injected				
	15	20·0	**3·66**	
	16	11·4	0·55	Mean = 0·54
	17	11·6	0·53	
BAL injected				
	18	27·5	**4·73**	
	19	12·2	0·73	
	20	10·6	0·49	Mean = 0·49
	21	7·0	0·35	
	22	13·0	0·39	

amount to 6·2, 4·95, 5·55, 4·0, 5·2, 1·59 and 4·58 mg. BAL corresponding to the excretion during the 24 hr. following injection of 21, 16·5, 18·5, 13, 17, 5 and 13% respectively of the amount of BAL injected.

The pre-injection samples of urine gave a negative nitroprusside reaction for thiol groups, the reaction remaining negative even after treatment with KCN to reduce any disulphide groups present. The 24 hr. specimen obtained following the injection of BAL gave in every case a strongly positive reaction for thiols. It cannot be claimed from these experiments that the rise in the iodine titre of the urine, following the injection of BAL, is due entirely to the excretion of thiols, but the strongly positive nitroprusside reaction given by the post-injection urine samples indicates that thiols are being excreted in markedly increased amounts. On account of the

small volume of urine obtained, no attempt was made in these experiments to identify the nature of the thiol excreted.

A further point of interest that emerges from these experiments is the rapidity with which the disturbance of thiol-disulphide equilibrium in the body produced by the injection of BAL is restored to normal; in every case except one, the thiol excretion had fallen to within the normal range 24 hr. after the injection. Moreover in two of the experiments the animals received repeated injections. The short duration of the metabolic disturbance produced by BAL is in agreement with the rapid return to normal in the general condition of the animals. It must be stressed that the dose injected into these animals was large, varying from 83–132 mg. BAL/kg. body weight, corresponding roughly to the subcutaneous injection of 7 g. of BAL into a 70 kg. man.

A few experiments were also carried out with male rabbits, the urine being obtained by catheterization. The rabbits were first catheterized, using a No. 3 soft rubber catheter, and the bladder emptied. A sub-lethal dose of BAL was then injected intraperitoneally, and the bladder contents drawn off at intervals thereafter. As in the previous experiments the urine samples were titrated with 0·02N or 0·01N-iodine; they were also tested for the presence of albumin and for —SH by the nitroprusside reaction. The results of three experiments are shown in Table 5. In rabbits also there is a rapid rise in the iodine titre of the urine following injection of BAL, a substantial increase occurring during the first hour. It will be noted that the thiol excretion begins to fall again after 3–4 hr. Expressed in terms of BAL, and assuming that the pre-injection level of iodine-titrating substances other than BAL is maintained constant, a total of 10·33, 8·82 and 9·48 mg. BAL were excreted in 6 hr. in these three experiments, corresponding to 14, 13·5 and 12% respectively of the amount injected. A control experiment was carried out to determine whether the injection of thiodiglycol alone influenced the excretion of thiols in the urine; it was found that the intraperitoneal injection of 0·2 ml. affected neither the iodine titre nor the nitroprusside reaction of the urine.

In each experiment the injection of BAL resulted in a transient albuminuria. In Exp. 390 a trace of albumin was present in the pre-injection specimen; this was probably brought about by struggling. The short duration of the albuminuria in these animals suggests that it is more the result of the vaso-dilator action of BAL on the renal vessels rather than the result of any serious damage to the glomeruli. In Exp. 409 the specimen obtained 1½ hr. after the injection was turbid and of a reddish brown colour; on centrifuging a clear, pale yellow supernatant was obtained, which contained a considerable quantity of albumin. The deposit was resuspended in 0·9% NaCl, examined microscopically and identified as red blood cells of normal appearance. No clots were present in the specimen, and from its homogeneous appearance it was felt that the blood was renal in origin and not due to trauma to the urethra or bladder. It would appear therefore that although the injection of BAL resulted in glomerular vaso-dilatation to such an

Table 5. *Urinary excretion of thiols after injection of BAL into rabbits*

Sample	Vol. urine (ml.)	Vol. 0·02N-I_2/10 ml. urine (ml.)	Vol. 0·02N-I_2/whole specimen (ml.)	Mean rate/hr. (ml. 0·02N-I_2)	Nitroprusside	Albumin
Exp. 390. Wt. rabbit = 2905 g. 11.0 a.m. catheterized; bladder emptied. 11.20 a.m. 75 mg. BAL (=26 mg./kg. body wt.) injected intraperitoneally. Catheterized again at 12.20, 2.20 and 5.20 p.m.						
Pre-injection	7·2	0·66	0·475	—	—	Trace
1 hr. after BAL	2·5	27·5	6·87	6·87	++++	+
3 hr. after BAL	8·3	11·1	9·2	4·6	++++	+
6 hr. after BAL	25·2	1·2	3·02	1·01	++	Trace
Exp. 409. Wt. rabbit = 1752 g. 10.40 a.m. catheterized; bladder emptied. 10.45 a.m. 65 mg. BAL (=37 mg./kg. body wt.) injected intraperitoneally. Catheterized again at 12.15, 1.45, 3.15 and 4.45 p.m.						
Pre-injection	11·2	0·56	0·63	—	—	—
1½ hr. after BAL	3·5	7·8	2·73	1·82	+++	++(+cells)
2 hr. after BAL	3·4	21·6	7·35	4·9	+++	Trace
4½ hr. after BAL	3·0	9·1	2·73	1·82	+++	—
6 hr. after BAL	5·5	4·1	2·26	1·51	++	—
Exp. 413. Wt. rabbit = 2129 g. 10.40 a.m. catheterized; bladder emptied. 10.45 a.m. 80 mg. BAL (=38 mg./kg. body wt.) injected intraperitoneally. Catheterized again at 12.15, 2.45 and 4.45 p.m.						
Pre-injection	4·6	1·0	0·46	—	—	—
1½ hr. after BAL	7·0	10·25	7·18	4·78	++++	Trace
4 hr. after BAL	4·0	18·60	7·44	2·97	++++	+
6 hr. after BAL	4·0	5·4	2·16	1·08	++	Trace

extent as to allow the passage of considerable quantities of protein, and possibly also of red blood cells, into the glomerular filtrate, it did not cause any detectable haemolysis.

DISCUSSION

The experiments described here show that the BAL treatment of lewisite burns in rats is followed by a marked increase in the elimination of arsenic in the urine during the succeeding 24 hr.; this increased excretion of arsenic is observed even when treatment with BAL is not begun until 1 hr. after contamination. The faecal excretion of arsenic does not appear to be significantly influenced by BAL. The severe diarrhoea invariably present in the control animals, however, was completely prevented by BAL treatment.

Analyses of the excised, contaminated skin showed that treatment with BAL causes a reduction in the amount of arsenic present in the skin 48 hr. after contamination. This finding is in agreement with the ability of BAL to prevent vesication in man after penetration of the skin by the lewisite.

No attempt was made at the time of this work to isolate or identify the derivative of lewisite excreted in the urine. It should be noted however that the subcutaneous injection into rats of the lewisite-BAL compound, prepared *in vitro*, is also followed by the urinary excretion of a large quantity of arsenic.

The disturbance of thiol-disulphide equilibrium caused by the subcutaneous injection of large amounts of BAL into rats or rabbits is rapidly re-adjusted by the excretion in the urine of an unidentified thiol. Although in rabbits a transient albuminuria accompanies the excretion of this thiol no evidence has been obtained of any serious or permanent functional damage to the kidney. A brief preliminary review of this work has already appeared (Peters, Stocken & Thompson, 1945).

SUMMARY

1. Experiments are described showing that the treatment of lewisite burns in rats by 2:3-dimercaptopropanol (British Anti-lewisite, BAL) is followed by a marked increase in the urinary excretion of arsenic during the succeeding 24 hr., amounts up to 33% of the arsenic applied to the skin (as lewisite) being recovered in the 24 hr. urine sample.

2. This increased excretion of arsenic is observed even when treatment with BAL is not begun until 1 hr. after contamination.

3. The severe diarrhoea and passage of mucus shown by control animals were completely prevented by BAL treatment.

4. Treatment with BAL caused a diminution in the amount of arsenic left in the skin 48 hr. after contamination.

5. The subcutaneous or intraperitoneal injection of large amounts of BAL into rats or rabbits is rapidly followed by the excretion of an unidentified thiol (or thiols) in the urine. In rabbits the thiol excretion after a single dose of BAL begins to fall off after 3–4 hr., but is still above the pre-injection level after 6 hr.

6. In rabbits a transient albuminuria accompanies the excretion of this thiol, but no evidence has been obtained of any serious or permanent functional damage to the kidney.

This work was carried out as part of a programme of extra-mural research for the Ministry of Supply under the direction of Prof. R. A. Peters, M.C., F.R.S. Our thanks are due to the Chief Scientific Officer, Ministry of Supply, for permission to publish, to Miss M. R. Kempson and Mr C. Dear for skilled technical assistance.

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The Effect of Phytic Acid on the Absorption of Calcium and Phosphorus

3. IN CHILDREN

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(*Received* 12 *March* 1946)

In children the demand for calcium is particularly great and since their diet may contain considerable amounts of phytate, which is known to impair calcium absorption in adults (McCance & Widdowson, 1942) and in infants (Hoff-Jørgensen, Andersen, Begtrup & Nielsen, 1946), we have investigated the effect of a diet rich in phytate on the absorption of calcium and phosphorus in two children aged 10 years.

EXPERIMENTAL

Subjects

Both children were admitted to hospital complaining of bronchial asthma. At the time of the experiment their condition had been cured, and they were detained at hospital only for the sake of the experiment. Particulars of the subjects are given in Table 1.

Table 1. *Particulars of subjects*

Subject	Date of Birth	Date of Admission	Weight (kg.)	Complaint on admission	Treatment
J.T.	18. ix. 34	30. xii. 44	15. iii. 45 27·6 8. iv. 45 27·7 30. iv. 45 27·6 20. v. 45 27·9 31. v. 45 28·0	Bronchial asthma	From 30. i. 45: 5000 i.u. vitamin A, 1000 i.u. vitamin D_2 and 30 mg. ascorbic acid daily. 15 treatments with u.-v. light
M.H.J.	2. vi. 34	22. i. 45	15. iii. 45 37·5 8. iv. 45 37·9 30. iv. 45 38·2 20. v. 45 38·5	Bronchial asthma	From 22. i. 45: as subject J.T., but no u.-v. light treatment

Diet

From the time of admission to the beginning of the experiment the subjects received the ordinary diet of the hospital. The daily calcium intake was about 1·2 g. During the whole experimental period the subjects were given 500 g. milk daily and no cheese. Care was taken that the diet in the different experimental periods, each of 5 days, was as uniform as possible.

Danish rye bread and Danish rye vita (both of which according to the law are baked from flour made from 85 parts of rye flour (98% extraction) and 15 parts of bran (80–98% extraction), wheat bread baked from 100% wheat flour and oatmeal porridge were given during the phytate periods.

During the periods with a low intake of phytate the bread was made from sifted rye (70% extraction). This type of bread, made with leaven, is phytate-free. A small amount of wheat bread was given as wheat rolls (80% extraction), and the porridge was prepared from barley grit which had been soaked in water overnight to ensure complete hydrolysis of the phytate. Milk and porridge (250 g.) were given at breakfast, and 250 g. milk and most of the bread at lunch. As an example of the composition of the diet during an experimental period of 5 days the data for the subject M.H.J. during period 4 are given in Table 2. With J.T. a further experiment was made (period 10) in which a phytate-free diet with 100 g. milk only was given daily for 5 days. The total calcium intake in this period thereby roughly corresponded with the intake of 'absorbable' calcium during the periods in which a diet rich in phytate was given.

Collection and analysis of excreta

The faeces of each subject were collected during each period in a covered glass bowl. The urine was collected under toluene after acidification with strong HCl. Carmine (1·0 g.) was used as a faecal marker to separate periods.

The analyses of food, faeces and urine were carried out as previously described (Hoff-Jørgensen, 1946).

Table 2. *Composition of the diet of subject M.H.J. during the 4th period*

Food	Intake Total (g.)	Ca (mg./100 g.)	Total P (mg./100 g.)	Phytate-P (mg./100 g.)
Rye bread	520	42	296	61
Wheat bread	400	39	236	103
Rye vita	275	68	432	300
Porridge (oat)	2125	12	58	42
Panade (rye)	1650	13	39	9
Sauce	150	9	14	
Milk	2500	117	97	
Butter	225	21	15	
3 eggs	290	81	226	
Meat	200	20	194	
Meat balls	300	14	131	
Sausage	50	6	110	

Food	Intake Total (g.)	Ca (mg./100 g.)	P (mg./100 g.)
Liver paste	125	9	140
Garfish	50	27	180
Rose-hip gruel	400	11	26
Fruit juice	500	6	5
Sago gruel	450	8	17
Apples	300	5	15
Potatoes	600	4	31
Peas	100	15	27
Carrots	200	30	44
Leeks	100	23	15
Cabbage	150	42	18
Jam	100	8	12
Water	1000	9	0

Table 3. *Retention of* Ca *and* P *from diets rich and poor in phytate*

Subject	Periods of 5 days No.	Dates	Intake of Ca (mg.)	Total P (mg.)	Phytate-P (mg.)	Excretion of Ca in Urine (mg.)	Ca in Faeces (mg.)	P in Urine (mg.)	P in Faeces (mg.)	Phytate-P in faeces (mg.)	Retention of Ca (mg.)	P (mg.)
M.H.J.	1	15. iii–20. iii	4,825	7,690	0	140	3,335	4,080	2,685	0	1,350	925
	2	24. iii–29. iii	4,531	7,472	70	144	3,525	4,455	2,115	0	862	902
	3	3. iv– 8. iv	4,415	7,525	40	112	3,270	4,015	2,280	0	1,033	1,230
	1+2+3		13,771	22,687	110	396	10,130	12,550	7,080	0	3,245	3,057
	4	10. iv–15. iv	4,730	10,396	2,596	122	5,020	4,230	4,285	1,025	−412	1,881
	5	17. iv–22. iv	4,575	9,860	2,215	134	4,085	5,310	3,125	765	356	1,425
	6	25. iv–30. iv	4,610	10,540	2,434	141	4,110	4,805	3,770	885	359	1,965
	4+5+6		13,915	30,796	7,245	397	13,215	14,345	11,180	2,675	303	5,271
	7	1. v–6. v	4,706	7,725	125	182	2,940	3,825	2,385	45	1,584	1,515
	8	8. v–13. v	4,485	7,560	0	126	3,340	3,535	3,165	0	1,019	860
	9	15. v–20. v	4,635	7,644	40	124	3,060	3,890	2,410	0	1,451	1,344
	7+8+9		13,826	22,929	165	432	9,340	11,250	7,960	45	4,054	3,719
J.T.	1	15. iii–20. iii	4,788	7,581	0	89	3,120	4,050	2,040	0	1,579	1,491
	2	24. iii–29. iii	4,494	7,398	65	93	3,400	2,785	2,655	80	1,001	1,958
	3	3. iv–8. iv	4,622	7,676	42	172	2,955	3,965	2,680	0	1,495	1,031
	1+2+3		13,904	22,655	107	354	9,475	10,800	7,375	80	4,075	4,480
	4	10. iv–15. iv	4,720	10,660	2,425	116	5,205	5,080	3,650	798	−601	1,930
	5	17. iv–22. iv	4,530	10,210	2,215	143	4,175	4,340	3,900	865	212	1,970
	6	25. iv–30. iv	4,615	10,300	2,430	102	4,385	4,525	4,125	716	128	1,650
	4+5+6		13,865	31,170	7,070	361	13,765	13,945	11,675	2,379	−261	5,550
	7	1. v–6. v	4,565	7,386	112	162	2,870	3,225	2,560	32	1,533	1,601
	8	8. v–13. v	4,430	7,410	0	98	2,545	3,060	3,005	0	1,787	1,345
	9	15. v–20. v	4,585	7,630	40	120	3,200	3,560	2,810	0	1,265	1,260
	7+8+9		13,580	22,426	152	380	8,615	9,845	8,375	32	4,585	4,206
	10	26. v–31. v	2,116	5,418	0	161	3,060	2,240	1,740	0	−1,005	1,438

RESULTS

The results of the experiments are given in Table 3.

DISCUSSION

Table 3 shows that calcium absorption was greatly reduced during the phytate periods. It is possible, however, that if the children had been kept on the diet rich in phytate for a longer period their calcium absorption might have gradually increased. Thus it is seen that the calcium absorption from a diet rich in phytate was considerably lower during the first 5 days' period than in the following periods. In both subjects, however, the increase in calcium absorption did not seem to continue beyond the second phytate period. Hence our results indicate

that in our subjects the 'adaptation' to conditions less favourable for calcium absorption was already complete after 7 days.

The composition of the calcium phytate precipitated in the intestine is probably $C_6H_8O_{24}P_6Ca_5$ (Hoff-Jørgensen, 1944) and therefore 1·0 g. of phytate-P should combine with 1·075 g. Ca to make the slightly soluble pentacalcium phytate. We therefore may estimate that in the phytate period 4 (subject J.T.) $2{\cdot}425 \times 1{\cdot}075 = 2{\cdot}61$ g. Ca was precipitated as calcium phytate and that $4{\cdot}720 - 2{\cdot}607 = 2{\cdot}013$ g. had not combined with phytate. In period 10 the intake of Ca from a phytate-free diet was 2·116 g. Therefore, if calcium phytate were completely unabsorbable we should have expected nearly the same calcium balance in period 10 as in period 4. The balance, however, was somewhat more positive in period 4; this indicates that a small part of the calcium which was precipitated as calcium phytate was absorbed.

Both the absorption and the retention of phosphorus were considerably greater on a diet rich in phytate than on a diet poor in phytate. The reason is presumably the same as that already considered in the discussion of the experiments with puppies (Hoff-Jørgensen, 1946).

The amount of phytate hydrolyzed in the intestines of the two children was about 70% of that in the diet, and some of the split phosphate may have been absorbed.

SUMMARY

1. Two boys aged 10 years received during three periods of 5 days a diet poor in phytate. A diet rich in phytate was then given for three similar periods, and finally the first diet was again given. The diets contained 500 g. milk and about 0·9 g. Ca daily.

2. The effect of phytate was: (*a*) a great reduction in the absorption and retention of calcium; (*b*) an increase in the absorption and retention of phosphorus.

The above work was part of the programme of the Cereal Committee of the Academy of Technical Sciences, Copenhagen.

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Effects of Vitamin A Deficiency in the Rabbit

1. ON VITAMIN C METABOLISM. 2. ON POWER TO USE PREFORMED VITAMIN A

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(*Received* 8 *March* 1946)

The level of vitamin A in the blood and that of ascorbic acid in the aqueous humour of rabbits during vitamin A deficiency and cure have been investigated in connexion with detailed studies of the effect of vitamin A deficiency on the eye. Although they have been obtained with relatively few animals, the results are reported since the main experiment has now ended. They suggest two conclusions; first that the ascorbic acid of the aqueous humour is lowered by vitamin A deficiency and second that prolonged deficiency impairs either absorption and/or storage of vitamin A. This second conclusion is based on the fact that dosage with vitamin A in oil does not bring the blood vitamin A level back to normal. Subsequent feeding with cabbage will do so although the amount of carotene given is approximately equal to the amount of vitamin A fed previously.

EXPERIMENTAL

The rabbits used were of mixed Dutch stock. Either the young were put on the diet at weaning (5–6 weeks) or the nursing does were fed the deficient diet and this was continued to the young afterwards. In some cases the young rabbits were given a single dose of 1000 i.u. of vitamin A before being started on the diet in order to try to reduce the deaths among the young deficient animals and to obtain a gradual rather than an acute onset of deficiency. The effect of such a dose can be seen by comparing Figs. 1 and 2. The litter whose growth is shown in Fig. 2 was given a starting dose of vitamin A, while that shown in Fig. 1 was not. It can be seen that the fall in growth rate due to deficiency occurs some months earlier in litter 1 (rabbits 40, 41) than in litter 2 (rabbits 50, 51).

Diet. A diet of crushed oats plus 1·5% of powdered chalk mixed and made just damp with water was fed *ad libitum*. The chalk was added to bring the calcium : phosphorus ratio of the oats near unity to diminish the need for vitamin D.

Rickets has not been reported in rabbits and in general no vitamin D was given to the animals but some of the young which were put on the diet at 3 weeks of age were given one or two weekly doses of 200 i.u. of irradiated ergosterol. They showed no increase in growth rate. Water was freely available and each rabbit was given 5 mg. of ascorbic acid each day mixed in a spoonful of bran for convenience of measurement. A brief analysis of crushed oats is given to show that the diet appears to be adequate:

Digestible nutrients of crushed oats (*Bull. Minist. Agric., Lond.*, no. 48): protein (N × 6·25) 8·0%, oil 4·0%, carbohydrate 45%, CaO 0·14%, P_2O_5 0·81%; factors of the B complex in μg/100 g. of wet crushed oats (Burkholder, 1943):

Aneurin 113, riboflavin 8, nicotinic acid 75, biotin 9, pantothenic acid 75, pyridoxin 3, folic acid 220.

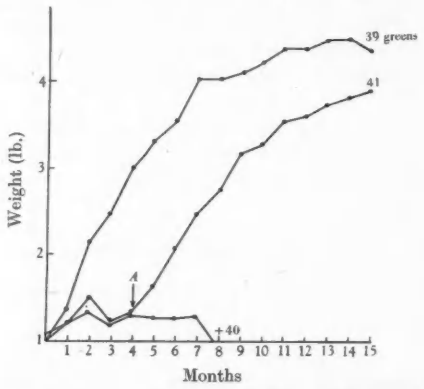

Fig. 1. Growth curves of three litter mates. No. 39 was fed greens throughout. No. 40 died after 8 months on the deficient diet. No. 41 was dosed with vitamin A concentrate after 4 months' deficiency.

The grown rabbits ate daily about 75 g. (dry) of the diet. Controls were given the same food with about 2 oz. of greens or a daily dose of about 500 i.u. of vitamin A by mouth. Those having vitamin A grew at about the same rate as those having greens and appeared healthy. The rabbits were weighed once a week and Figs. 1 and 2 show the growth curves of two litters both of which contain control and deficient animals.

Criteria of deficiency and cure. The deficiency was assessed partly by growth rate, partly by estimation of plasma vitamin A and partly by slit-lamp examination of the eyes. The earliest sign was a reduction in growth rate. The time after which this occurred depended upon the age at which the rabbit was started on the deficient diet. The younger the rabbit at the start the sooner it stopped growing. The next sign was a fall in plasma vitamin A. This might fall to 10–20 i.u. of vitamin A/100 ml. of plasma, which is about 10% of the normal value, before any eye changes could be seen with the slit-lamp. All rabbits were kept on the deficient diet until they showed either microscopically visible or macroscopic changes in their corneas of the type described by Mann, Pirie, Tansley & Wood (1946) and by earlier workers.

When eye signs of the deficiency were established the animals were given 500–1000 i.u. of vitamin A in oil by mouth each day in order to effect as rapid a cure as possible. The corneal lesions showed an immediate change and the cornea was usually normal in 1–3 weeks from the start of dosage with vitamin A. The rabbits also slowly began to put on weight. A rabbit was taken as cured when its eyes were normal, but in assessing the effectiveness of vitamin A in raising the plasma vitamin A level, dosage was continued for at least one month before plasma levels were determined and, in most cases, was continued for much longer periods.

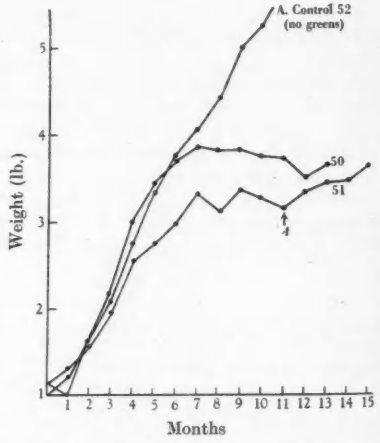

Fig. 2. Growth curves of three litter mates. All were given one dose *c.* 1000 i.u. of vitamin A at start of experiment (3 weeks old). No. 52 was given vitamin A concentrate throughout life. No. 50 was on deficient diet for 14 months and no. 51 was cured with vitamin A concentrate after 11 months' deficiency.

Estimation of vitamin A in plasma. Blood was taken before the morning feed from the marginal ear vein into citrated tubes. Plasma (3–5 ml.) were stored overnight in the ice-chest and the carotene and vitamin A were then estimated by a modification of the method, described by Yudkin (1941). Plasma (2 ml.) were pipetted into a 15 ml. test tube and 2·0 ml. 95% ethanol added drop by drop, the tube being well shaken after each addition. Light petroleum (b.p. 40–60°) (4 ml.) was added, the tube well corked and shaken by hand for 10 min. The layers were allowed to separate and 2·0 ml. of the petroleum layer was transferred to a colorimeter tube. Light petroleum (0·25 ml.) was then added and the carotene measured in a photo-electric colorimeter with a 440 mμ filter. After the petroleum had been evaporated off on a water-bath at 55–60° and then for a few seconds at 70° the residue was dissolved in 0·2 ml. of anhydrous chloroform, one drop of acetic anhydride was added and the tube placed in the colorimeter with the light intensity already adjusted (620 mμ filter). Carr-Price reagent (2 ml.) was added to the tube in position from an automatic pipette and the maximum temporarily steady galvanometer deflexion recorded. The tube was removed at

once and examined. The result was discarded if the solution was turbid. Galvanometer readings were converted to i.u. carotene and vitamin A/100 ml. of plasma by reference to calibration curves.

Estimation of ascorbic acid in aqueous humour. The aqueous humour was removed from the eye by inserting a fine glass capillary pipette through the margin of the cornea into the anterior chamber and allowing the fluid to run into the pipette under its own pressure. Cocaine was sufficient as an anaesthetic. The volume of aqueous humour was measured, it was usually about 0·2 ml., and an equal volume of 30% (w/v) metaphosphoric acid was added. The total volume was made up to 1·0 ml. and any small protein precipitate centrifuged down. The ascorbic acid was estimated by titration with 0·05–0·1 ml. of 2–6-dichlorophenol-indophenol from a microburette.

RESULTS

Effect of vitamin A deficiency on the ascorbic acid content of the aqueous humour. Thirteen deficient rabbits and four control rabbits fed on vitamin A in oil were investigated. In some cases a rabbit was used more than once but if the aqueous humour was withdrawn twice from the same eye an interval of several weeks was allowed for the composition of the aqueous humour to return to normal. Where

Table 1. *Ascorbic acid content of aqueous humour of rabbits*

(The mean value + its standard error are given in each series.)

(*a*)

Vitamin A-deficient (mg./100 ml.)	Vitamin A controls (mg./100 ml.)
16·8±1·3	24·7±2·3
Difference 7·9±2·6	

(*b*)

	Vitamin A-deficient		Vitamin A-deficient after cure	
No. of rabbit	Time deficient (months)	Ascorbic acid (mg./100 ml.)	Time of cure months	Ascorbic acid (mg./100 ml.)
51	10	16	6	24
				24
62	6	11	2	12
38	5	24	1	23
41	3½	15	2	28
			4	27
			11	34
45	1¾	20	3	35
Mean		17·3		24·8
		Difference 7·6±3·4		

there are two determinations on the same rabbit the mean of the two values has been used in calculating the results. The ascorbic acid of aqueous humour of five rabbits cured by dosage with vitamin A in oil was also estimated. Table 1 shows that there is a fall in ascorbic acid in the aqueous humour of vitamin A-deficient rabbits. The cured rabbits were too few to give a reliable assessment of comparative ascorbic acid values, since animals varied widely, but three-fifths showed an increase on cure and the averages for the series were remarkably like those for the deficient and normal control series. There seemed to be no relation between the severity of changes in the cornea judged by slit-lamp microscopy and the decrease in the aqueous humour ascorbic acid.

Sure, Theis & Harrison (1939) found that many tissues from vitamin A-deficient rats had less ascorbic acid than those from normal animals and Boyer, Phillips, Pounder, Jensen, Rupel & Nesbit (1942) found that vitamin A-deficient calves showed low plasma and tissue ascorbic acid and vitamin A-deficient rats excreted less ascorbic acid than normal controls. Both calves and rats are independent of an outside source of ascorbic acid and one can therefore conclude that it is ascorbic acid synthesis that is depressed in vitamin A deficiency. The rabbit is also independent of an outside source of ascorbic acid, but in all our experiments each rabbit was given *c.* 5 mg. of ascorbic acid each day mixed with its food. The fall in ascorbic acid in the aqueous humour during vitamin A deficiency therefore occurred while the animals were getting a small but probably adequate supply in their food. It might have been greater without this.

The concentration of ascorbic acid in the aqueous humour of normal rabbits has been estimated by many different workers and Bellows (1944) gives the average as 25 mg./100 ml. with a range of 13–50 mg./100 ml. The average is the same as the average value for our control series which were fed 5 mg. ascorbic acid each day. We have found that rabbits fed on green food usually have a higher concentration than this, the average being 35 mg./100 ml. of aqueous humour.

Factors affecting blood vitamin A levels in deficient and cured rabbits. The blood vitamin A value usually sank to 10–20% of the normal value before signs of deficiency could be detected in the eyes (Mann *et al.* 1946). Again, the number of animals is small, but a series of estimations were done on each. Six rabbits were used as controls on green food, two as controls on vitamin A in oil, twelve were made deficient and were then clinically cured with vitamin A in oil and five of these were subsequently fed greens. In setting out the results the cured rabbits have been divided into two groups, those that were 6 months or more deficient, and those deficient for a shorter period. Values for the cured rabbits were obtained at least one month after the start of dosage and, with the usual high dose, we felt that the vitamin A reserves of the animals should have been normal after this time. The eyes of the deficient animals were all normal 1–3 weeks after start of dosage with the vitamin.

Table 2. *Blood vitamin A of control and cured rabbits*

(The mean value + its standard error are given for each group.)

	No. of animals	Blood vitamin A (i.u./100 ml.)
1. Control animals given:		
(*a*) Vitamin A	2	109·5 ± 27·4
(*b*) Green food	6	118·5 ± 15·8
2. Animals cured with vitamin A:		
(*a*) 6 months or more deficient	6	55·8 ± 15·8
(*b*) Less than 6 months deficient	6	120 ± 15·8

Five rabbits which had been dosed with vitamin A in oil in order to cure their deficiency were then given *c.* 2 oz. of green food each day and their blood vitamin A was re-determined after 7–12 days. The average value of the blood vitamin A in these five rabbits was 70·8 i.u./100 ml. on vitamin A in oil and on greens it was 144·8 i.u./100 ml., an increase of 74·0 ± 13·2 i.u./100 ml. The amount of carotene in the daily green food was very kindly estimated for us by Dr T. Moore and found to be equivalent to *c.* 500 i.u. of vitamin A. The rabbits were therefore getting at least as much vitamin A when dosed with vitamin A in oil as they were when fed greens, but their blood vitamin A became much higher on the green food.

Wilson, Ahmed & Majumdar (1936) report that vitamin A-deficient rats respond better to vegetables than to carotene in oil. Here our rabbits responded to vegetables better than to vitamin A in oil, although in general the evidence, summarized by Gridgeman (1944), shows that animals use vitamin A more efficiently than carotene.

To see whether it was absorption from the gut or storage in the liver that was at fault, the vitamin A content of the livers of rabbits fed on the basal diet plus vitamin A in oil throughout life was compared with that of rabbits which had been made deficient and then cured, judged by disappearance of eye signs, by daily doses of vitamin A in oil. None of these rabbits were given green food at any stage of the experiment. Table 3 shows that, after prolonged deficiency, four rabbits still had a low liver vitamin A after 1½–6 months dosage with the vitamin. The results do not decide whether it is only absorption of vitamin A that is at fault after prolonged deficiency or whether both absorption and liver storage are impaired. The many factors, known to affect absorption of vitamin A (Moore, 1940; Gridgeman, 1944), cannot be discussed since they were not investigated.

Table 3. *Comparison of liver vitamin A of control rabbits and of those cured after varying periods of vitamin A deficiency*

No. of rabbit	Period of deficiency (months)	Period of dosage with vitamin A in oil (months)	Vitamin A in liver (i.u./g.)
52	0	16 (throughout life)	296
61	0	8 (throughout life)	183
41	3·5	11	212
54	3·5	1	200
51	10	6	52
62	6	2	44
25	6	1·75	22
38	5	1·5	5

It must be stressed that if the animals were fed the basal diet plus vitamin A in oil throughout life they grew normally and maintained a high plasma and liver vitamin A content. Vitamin A in oil solution was insufficient to restore the plasma vitamin A to a normal level only after a period of prolonged deficiency. Vitamin A is an unnatural constituent of the rabbit's food and such a 'secondary' deficiency would not occur under natural conditions. It is only in man that large daily doses of vitamin A in oil are likely to be given to cure vitamin A deficiency and these experiments are reported to show that at least in one animal species cabbage may be more effective than the isolated vitamin.

SUMMARY

1. The ascorbic acid content of the aqueous humour of vitamin A-deficient rabbits is decreased.

2. A small number of rabbits that were vitamin A deficient for 6 months or longer did not respond well to dosage with vitamin A in arachis oil. The level of vitamin A in their plasma and in their liver remained lower than normal.

3. Feeding with cabbage restored the plasma vitamin A to normal.

We acknowledge with pleasure much help from Mr D. J. Finney in the assessment and presentation of the results.

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The Influence of Adrenalectomy on Muscle Constituents

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(*Received* 4 *March* 1946)

The following studies were initially undertaken to investigate the quantitative relations of potassium changes in blood and muscle after adrenalectomy in the light of the work done on the nature of potassium accumulation (Boyle & Conway, 1941). In the course of the investigation the mean values of the known and quantitatively important substances in muscle and plasma were determined. These include Na, K, Ca, Mg, Cl, HCO_3, pH, water, inorganic phosphate, total phosphate, urea, and total molecular concentration in plasma; Na, K, Ca, Mg, Cl, water, carnosine, anserine, total phosphate, total acid-soluble phosphate, adenosinetriphosphate, phosphocreatine glucose-6-phosphate, glucose-1-phosphate, fructose-6-phosphate, fructose-1:6-diphosphate, triose phosphate, phosphoglyceric acid, and phosphopyruvic acid in muscle. The results were used to calculate the change in non-diffusible material in the muscle fibre as well as the sum of the electrical charges thereon (η and ϵ values) following adrenalectomy with a view to interpreting more fully the cation changes. While this was the primary object of the present study, the greatly altered concentrations of the hexose esters appear to provide results of interest with respect to the changes in the intermediary metabolism of carbohydrate.

METHODS

Treatment of animals

Albino rats, almost all of the Wistar strain, were used as experimental animals; young animals weighing about 70–100 g. were chosen as these showed more marked deficiency conditions than older rats, which have some accessory adrenal tissue not removed at operation and elaborating sufficient hormone to relieve the symptoms (Verzár, 1939). These rats were maintained on a low Na diet following adrenalectomy, controls being maintained on the same diet for a corresponding number of days.

Removal of glands. The rat was anaesthetized by means of an ether-soaked pad of cotton-wool in a large glass jar and tied out back upwards on a flat board. The hair of the back was then clipped short and a cut about 2–3 cm. long made across the middle of the back. The skin was then retracted and a small incision about 5 mm. long made through the muscles a little below the ribs. The gland was then located on the upper pole of the kidney and removed by quickly tearing away with forceps; no appreciable bleeding occurred in this method and consequently ligatures were unnecessary. After removal of both glands in this manner the severed tissues were stitched in position and the wound and adjacent skin region painted with diluted iodine solution. Following the operation the rats were maintained at a temperature of about 65° F. in a thermostatically controlled room. Four or five days after the operation they were taken for the blood and muscle analyses, but within that interval they occasionally died.

Sampling of blood and muscle. The estimations of the various muscle constituents were carried out on the leg muscles, excised immediately on killing the animal. Blood was collected by bleeding directly into a centrifuge tube (under light ether anaesthesia) except when required for pH, bicarbonate, and chloride determinations, when it was collected without exposure to air under liquid paraffin through a cannula in the abdominal aorta under ether anaesthesia; or when required for total molecular concentration determination, when a few drops were taken from the tail vein.

Chemical estimations. Inorganic. Sodium and potassium were estimated by colorimetric modifications (Boyle, Conway, Kane & O'Reilly, 1941) of the methods of Salit (1932) and Shohl & Bennett (1928) respectively, with preliminary ashing as described by Fenn & Cobb (1934). Magnesium was determined by a hydroxy-quinoline method (Cruess-Callaghan, 1935) and calcium was determined by the method of Kramer & Tisdall (1925) as modified by Clark & Collip (1925). Chloride and bicarbonate were estimated in plasma by micro-diffusion methods (Conway, 1939) and muscle chloride was estimated by a modification of the method described for amphibian muscle (Conway, 1939) involving the diffusion of the chloride from muscle slices into isotonic Na_2SO_4, removal of protein by sodium tungstate and H_2SO_4, and estimation of chloride in the filtrate by the micro-diffusion method. The *water content* was found by drying overnight at 105° in platinum crucibles prior to ashing for Na and K estimations. The pH of the blood was measured by means of the Beckman meter. In determining the total molecular concentration of the blood a new micro-diffusion technique was used requiring only 3–4 mg. for an individual determination, so that estimations could be carried out on the same rat before and after adrenalectomy by removing a few drops of blood from the tail vein. This method will be described in detail elsewhere. The results after adrenalectomy are given here as a percentage of the normal value.

Organic. Urea was estimated by the Conway (1939) method and carnosine and anserine were determined by the methods of Zapp & Wilson (1938). The various compounds occurring in the carbohydrate cycle were estimated after a fractionation procedure described in the following section, which is a combination and modification of the methods of Le Page & Umbreit (1943), Needham & van Heyningen (1935), Eggleton & Eggleton (1929) and Stone (1943).

Extraction of muscle and fractionation of extract

The rat is killed by a sharp blow on the head and about 2–3 g. muscle (excised within a few seconds of the animal's death) are dropped into 25 ml. ice-cold trichloroacetic acid in a mortar containing a little quartz sand and a pestle (all chilled in refrigerator, and weighed), ground immediately, reweighed, and then ground very finely. The solution is poured into a chilled 25 ml. centrifuge tube and quickly spun, using ice-water to jacket the tube, then poured through a small dry filter into a second chilled 25 ml. tube accurately graduated at 15 ml. until the graduation is reached, the remainder of the extract being filtered into another dry tube and left aside in the refrigerator. 1 ml. 25% Ba acetate is added to the solution, which is then neutralized by addition of conc. NaOH (phenolphthalein indicator), the tube and contents being kept cool by crushed ice. The end-point is attained if necessary with 10% trichloroacetic acid or dilute NaOH and the tube is allowed to stand for 1 hr. in the refrigerator, then centrifuged (the tube again being jacketed with ice-water) and washed once with about 2 ml. of an ice-cold Ba trichloroacetate solution (neutral) prepared by carrying out a blank procedure. The Ba precipitate, which gives fraction 1, is left aside in the refrigerator. (From the point of excision of the muscle until the addition of the Ba acetate all the procedures must be carried out as quickly as possible, all necessary reagents, chilled tubes, ice-water, etc., being previously prepared and ready to hand. With some practice it is possible to precipitate fraction 1 within 10 min. of the death of the animal.)

To the centrifugate and washings in a 150 ml. beaker, 5 vol. of ice-cold 95% (w/v) aqueous ethanol (neutral) are added. The end-point is adjusted to neutral if necessary and the mixture allowed to stand in the refrigerator for 3 hr. The precipitate which forms is collected by centrifuging successive portions in a 50 ml. pointed centrifuge tube at high speed (3000–4000 r.p.m.) washed with ice-cold 80% (w/v) aqueous ethanol (neutral) and left aside in the refrigerator. This precipitate gives fraction 2.

The centrifugate and washings from this procedure are concentrated to a small volume *in vacuo* at 35–40° (fraction 3).

The Ba and ethanol precipitates are each dissolved in about 2 ml. 2N-HCl and the Ba is removed by adding 1–2 drops conc. H_2SO_4 and centrifuging. The centrifugates and washings ($BaSO_4$ precipitates are washed with 1 ml. distilled water) are taken in specially graduated tubes, neutralized with strong NaOH solution, and made up to 5 ml. The temperature is kept low during addition of H_2SO_4 and neutralizing by immersing the tubes in crushed ice. Fraction 3 is concentrated to about 2–3 ml., the Ba removed by H_2SO_4, neutralized, and made up to 5 ml.

The following estimations on the various fractions are then carried out as required:

Original extract. Total phosphate, phosphocreatine, triose phosphate, phosphoglyceric acid.

Fraction 1. Total phosphate, adenosinetriphosphate, fructose-1:6-diphosphate, pentose.

Fraction 2. Total phosphate, total hexose esters, glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, phosphopyruvic acid, pentose.

Fraction 3. Total phosphate (not further fractionated).

The recovery of total acid-soluble phosphate from the three fractions is about 95%.

Estimation of constituents in fractionated muscle extract

Inorganic phosphate was determined by the method of Fiske & Subarrow (1925); phosphocreatine and total phosphate were measured by this method after hydrolysis and wet ashing procedures respectively. Adenosinetriphosphate was measured by the increase in free phosphate after 7 min. hydrolysis in N-HCl at 100° (Lohmann, 1929). Phosphoglyceric acid was estimated by the Rapoport method (1937) and triose phosphate as phosphoglyceric acid after oxidation by iodine (Meyerhof & Junowicz-Kocholaty, 1943). Fructose was estimated by Roe's method (1934) and pentose by the method of Mejbaum (1939). Phosphopyruvic acid was determined by measuring the pyruvic acid set free on hydrolysis for 60 min. at 100° in N-HCl (Lohmann & Meyerhof, 1934) by the method of Friedemann & Haugen (1943).

Hexose esters. The total hexose esters were determined by measuring the total phosphate remaining in fraction 2 after the residual unhydrolyzed phosphocreatine had been removed by HNO_3 and ammonium molybdate (Sacks, 1944) an allowance being made for the small amount of phosphopyruvic acid present. The increase in reducing value of the fraction after 7 min. hydrolysis in N-HCl at 100° affords a method of estimating the glucose-1-phosphate present, since glucose-6-phosphate and fructose-6-phosphate are already reducing without hydrolysis. Glucose-1-phosphate on hydrolysis had a reducing value 66·4% that of glucose (Le Page & Umbreit, 1943). The reducing value was determined by the Folin & Malmros (1929) method; the Shaffer & Hartman (1921) method as modified by Somogyi (1926) and the delicate method of Florkin & Bosson (1929) were also tried but were less suitable for our purpose. Fructose-6-phosphate was estimated by determining the fructose present in this fraction. The difference between the total hexose esters and the glucose-1-phosphate + fructose-6-phosphate then gives a figure for glucose-6-phosphate.

In almost all cases the methods referred to were modified in details. In all the colorimetric methods extinction curves were prepared from suitable standard solutions of purified salts; the Pulfrich photometer or the Spekker photoelectric absorptiometer was used to measure the extinctions. The micro-cell of the Pulfrich photometer, which gives a 5 cm. layer with a volume of about 1·5 ml., was found particularly useful for low concentrations.

RESULTS

Muscle

The results for muscle are summarized as mean values in Table 1. From an inspection of Table 1 and considering the difference in the mean values and the standard deviations it would appear that the following substances are definitely increased after adrenalectomy (Table 2): potassium, magnesium, phosphocreatine, glucose-1-phosphate. Definite decreases are shown by sodium, glucose-6-phosphate, and fructose-6-phosphate, and probable decreases by total phosphate, chloride, and fructose-1:6-diphosphate.

The most striking percentage changes are shown by glucose-1-phosphate, glucose-6-phosphate, and

Table 1. *Summary of data for normal and adrenalectomized rat muscle*

(The figures in brackets give the number of experiments followed by the total number of rats in the group. Where no second figure is given in the brackets one rat only was used for each experiment.)

	Normal		Adrenalectomized	
	Mean (mmol./kg.)	S.E. of mean	Mean (mmol./kg.)	S.E. of mean
Potassium	101 (8, 15)	1·9	118·5 (15, 31)	1·3
Sodium	27·1 (10)	1·1	19·7 (19)	0·9
Calcium	1·56 (5, 7)	0·04	1·54 (6, 18)	0·06
Magnesium	11·0 (8, 14)	0·3	12·4 (8, 23)	0·4
Chloride	16·2 (5)	1·5	14·2 (9)	1·3
Total P	82·9 (7)	0·9	79·5 (11)	0·8
Acid-soluble P	61·1 (12)	0·8	60·7 (7)	1·6
Phospho-creatine-P	24·4 (11)	0·4	27·7 (12)	0·4
Adenosine-triphosphate-P	19·5 (8)	0·8	19·9 (9)	0·5
Total hexose monophos-phate-P	10·04 (7)	0·87	5·67 (13)	0·46
Glucose-6-phosphate-P	6·42 (7)	0·85	2·06 (11)	0·16
Glucose-1-phosphate-P	2·74 (8)	0·21	3·68 (15)	0·19
Fructose-6-phosphate-P	0·94 (9)	0·06	0·34 (7)	0·03
Fructose-1:6-di-phosphate-P	0·46 (11)	0·06	0·35 (7)	0·05
Triose phosphate + phosphoglyceric acid-P	3·55	—	3·55	—
Phosphopyruvic acid-P	0·073 (6)	0·004	0·077 (1)	—
Carnosine	1·36 (5, 14)	0·11	1·10 (7, 19)	0·12
Anserine	20·3 (5, 14)	4·9	23·5 (7, 19)	—
H_2O content (%)	76·8 (6)	0·3	77·4	0·3

In the above table millimolar values of adenosinetriphosphate and fructose-1:6-diphosphate are obtained by dividing the listed P values by 3 and 2 respectively.

Table 3. *Summary of data for normal and adrenalectomized rat plasma*

(The figures in brackets have the same denotation as for Table 1)

	Normal		Adrenalectomized	
	Mean (mmol./l.)	S.E. of mean	Mean (mmol./l.)	S.E. of mean
pH (38°)	7·32 (4)	0·06	7·14 (4)	0·07
K	5·91 (9, 13)	0·18	9·16 (9, 13)	0·40
Na	138 (11)	1·8	128 (11)	3·0
Ca	3·11 (5, 8)	0·12	2·82 (5, 15)	0·10
Mg	1·46 (8, 14)	0·06	2·03 (8, 23)	0·09
Cl	110 (9)	0·9	104 (5)	1·0
HCO_3 (as CO_2)	22·4 (9)	0·5	20·1 (7)	0·3
Inorganic P	2·17 (7)	0·05	2·72 (7)	0·21
Total P	3·89 (7)	0·15	4·54 (7)	0·20
Urea	6·7 (7)	0·8	8·3 (7)	1·0
Total molar concentration taking normal value as 100	100 (7)	5·8	86 (7)	5·4
H_2O content (g./100 g.)	92·4	0·1	92·3	0·3

Table 4. *Substances in plasma increased or decreased after adrenalectomy*

	% increase		mmol./l. increase	
	Mean	Median	Mean	Median
Potassium	55·0	54·7	3·25	3·08
Magnesium	39·0	44·3	0·57	0·62
Urea	23·9	20·0	1·6	1·2
Total phosphate	16·7	19·3	0·65	0·73
Inorganic phosphate	25·4	29·4	0·55	0·62
	% decrease		mmol./l. decrease	
	Mean	Median	Mean	Median
Sodium	7·3	5·8	10·0	8·0
Chloride	5·5	4·6	6·0	5·0
Bicarbonate	10·3	11·1	2·3	2·5
Calcium	9·3	15·1	0·29	0·47

Table 2. *Substances in muscle increased or decreased after adrenalectomy*

	% increase		mmol./kg. increase	
	Mean	Median	Mean	Median
Potassium	17·3	14·7	17·5	15·0
Magnesium	12·7	16·2	1·4	1·8
Phosphocreatine	13·5	13·5	3·3	3·3
Glucose-1-phosphate	34·3	19·8	0·94	0·57
	% decrease		mmol./kg. decrease	
	Mean	Median	Mean	Median
Sodium	27·3	27·1	7·4	7·3
Glucose-6-phosphate	67·9	71·6	4·36	4·71
Fructose-6-phosphate	63·8	67·4	0·60	0·64

fructose-6-phosphate, whilst the following substances show no appreciable change: adenosinetriphosphate, total acid-soluble phosphate, calcium. The distributions for substances definitely increased or decreased are shown in Fig. 1.

Plasma

The results are summarized in Table 3. Here also, on considering the difference of the means and the standard deviations, we may list definite increases in potassium, magnesium, and urea, and very probable increases of total phosphate and inorganic phosphate.

The following show a definite decrease: sodium, chloride, bicarbonate; and probable decrease of calcium (Table 4). The distributions for substances definitely increased or decreased are shown in Fig. 2.

Calculation of η and ϵ values

Here η = the 'idiomolar' value or sum in mmol. of the non-diffusible constituents in a number of muscle fibres which, for the normal animal, contained 1 l. of water.

For the computation of apparent values of η and ϵ we have the following equations (Boyle & Conway, 1941):

$$\eta/V = c - k_1 - \Sigma d_1, \quad (1)$$

$$\epsilon/V = k_1 - \Sigma d_1, \quad (2)$$

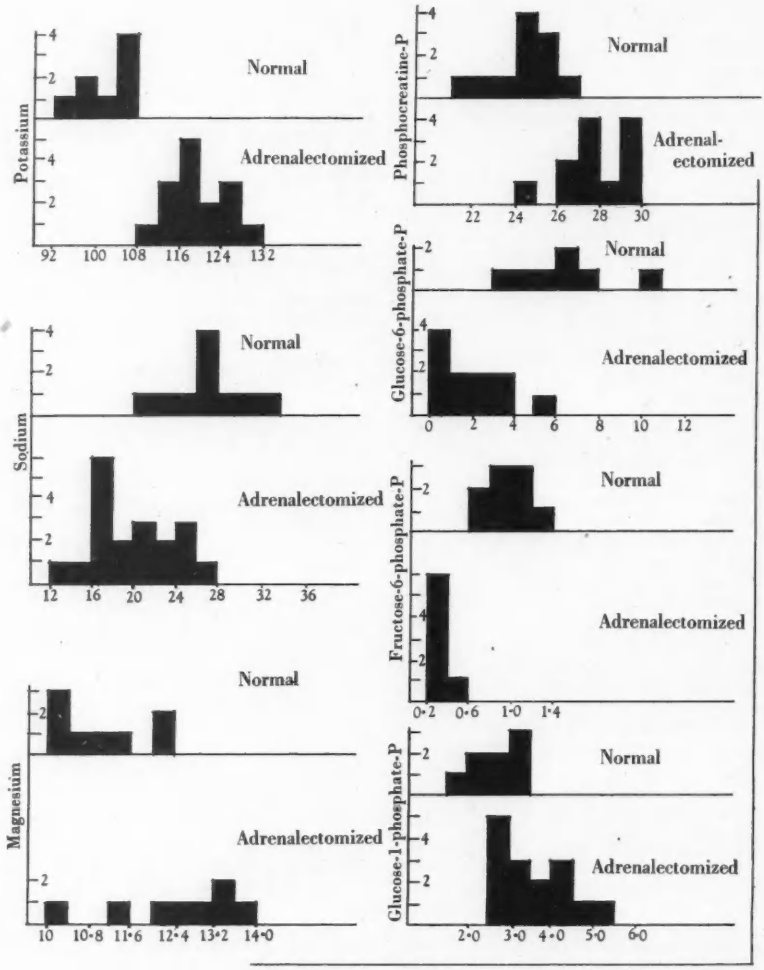

Fig. 1. Frequency distributions of muscle data for normal and adrenalectomized rats. The mean values are given in Table 1. Abscissae (class intervals) are given in mmol./kg., and ordinates as numbers of observations.

ϵ = the electrostatic equivalent of η or the m-equiv. of base required to satisfy the surplus negative charges on η.

where V = volume of fibre water (taken as 1·0 for normal muscle, and in accordance with the above definition of η); c = total concentration of ions and

non-diffusible non-electrolytes in the plasma water; k_1 = mmol. K/l.; and Σd_1 = sum of the diffusible anions/l. fibre water.

To obtain values for k_1 and Σd_1 it is necessary to determine the extracellular space in muscle. This may be done by assuming a Donnan relation for K and Cl across the fibres. Here the experiments of Wilde (1945) may be considered. He showed for nephrectomized rats that the general relationships predictable from the Conway theory (Conway & Boyle, 1939; Boyle & Conway, 1941) held with respect to K, Cl and water exchanges in the muscle of live rats, but he found the product of K and Cl concentrations within the fibres to be only 0·5–0·6 times the product outside. The extracellular space in the tissue was determined by inulin. Now the inulin space does not give an exact measure of the Cl in the tissue outside the fibres, since inulin does not penetrate the red corpuscles in the capillaries or small blood vessels. For excised frogs' sartorii (Boyle *et al.* 1941) we have found that 2·3% of the whole tissue could be reckoned as

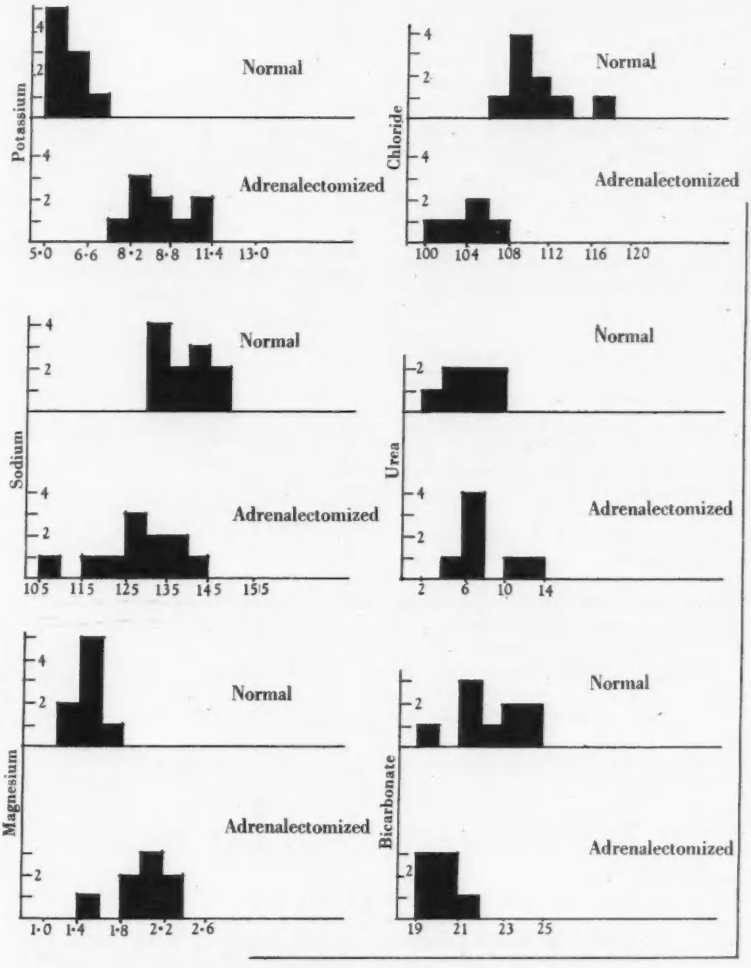

Fig. 2. Frequency distributions of plasma data for normal and adrenalectomized rats. The mean values are given in Table 3. Abscissae and ordinates as in Fig. 1.

blood. Assuming a similar figure for mammalian excised muscle, the allowance necessary causes a very appreciable diminution in the apparent concentration of Cl within the fibre. When it is taken into account that the Cl in the interspace water is somewhat higher than in blood water (just as the Na and K to maintain a Donnan relation across the capillary walls may be expected to be lower) and may be represented from the available data as about 1·05 times, then the ratio of the products of K and Cl outside and inside the membrane becomes approximately 0·9. If the blood in the mammalian muscle were 4·6 % instead of 2·3 % of the whole we would obtain a ratio of 1·1. We may therefore suppose that the ratio is certainly higher than 0·6, and is at or near to 1·0, thus agreeing with the findings for frog muscle immersed in Ringer solutions with high KCl content (Boyle & Conway, 1941).

In calculating s or the interspace we may now consider the equation

$$k_1 l_1 = k_2 l_2, \qquad (3)$$

where k_1, l_1 and k_2, l_2 are the K and Cl concentrations (mmol./l.) in the fibre and interspace water respectively.

The product $k_1 l_1$ may be expanded to

$$\frac{(k_0 - s k_2)}{(0{\cdot}768 - 0{\cdot}99s)} \times \frac{(l_0 - s l_2)}{(0{\cdot}768 - 0{\cdot}99s)},$$

where k_0 and l_0 are the mmol./kg. K and Cl muscle, 0·768 and 0·99 being the water content of 1 kg. tissue and of interspace fluid respectively.

The product $k_2 l_2$ may be taken as the same numerically as the similar product for plasma water, which from Table 1 (and taking the specific gravity of plasma as 1·03) may be written $6{\cdot}2 \times 116$. The value of s may then be calculated as 0·107. In a similar way for the adrenalectomized animal s is found to be 0·09.

From such data and similar calculations for adrenalectomized muscle the values of Table 5 are deduced, and from equations (1) and (2), the values of η and ϵ are 147 and 146 for the normal rat muscle and 120 and 175 for that of the adrenalectomized rat. In the calculation the V value for adrenalectomized muscle is based on the assumption that the same number of fibres are associated with the same dry weight of tissue. It will thus appear that there is a fall of 27 in the 'idiomolar' value and a rise of 29 in its electrostatic equivalent.

Computing η values in muscle from the direct analyses, and omitting anserine (the determination of which is not very exact), there appears a fall of about 9–10 mmol./kg. of fibre water due to the fall in total hexose esters and in Na over the small gains of phosphocreatine and magnesium. The remaining 17 mmol. would remain unaccounted for by the direct analyses.

Table 5. *Concentrations in the 'fibre water'*

(Symbols as in text)

	Normal	Adrenalectomized
k_1	152 m-equiv./l.	170 m-equiv./l.
l_1	4·7 m-equiv./l.	6·1 m-equiv./l.
$[HCO_3]$	0·9 m-equiv./l.	1·2 m-equiv./l.
s	0·107	0·09
W	0·662	0·688
Dry wt.	0·232	0·226
V	1·00	1·07
η	147	120
ϵ	146	175

(V is calculated on the basis that the same number of fibres after adrenalectomy are associated with the same dry weight of tissue. Thus W above (the water content of the fibres/kg. muscle) for the adrenalectomized rat is then multiplied by $\frac{232}{226}$ and $V = \frac{0{\cdot}688}{0{\cdot}662} \times \frac{232}{226} = 1{\cdot}07$.)

DISCUSSION

Inorganic changes. With regard to the inorganic constituents of the plasma, apart from the well-known changes in K and Na content, the mean increase in Mg is noteworthy and is accompanied by a similar increase in muscle Mg; the ratio of increase is similar to that of K. Total P and inorganic P appear also to be increased, with a fall in the bicarbonate and pH.

In muscle the mean changes observed by us for K, Na, P (total), Cl, and water follow closely those of Harrison & Darrow (1928). In addition we have shown a mean increase in Mg of 16–17 %—practically the same relative increase as for the potassium. A marked increase in muscle Mg does not appear in the experiments of Buell & Turner (1941). This rise in muscle Mg may contribute to the asthenia after adrenalectomy, since it has been shown that Mg inhibits adenosinetriphosphatase (Greville & Lehmann, 1943).

Changes in η and ϵ. η, or the total non-diffusible substances falls from 147 to 120 after adrenalectomy, and ϵ, the electrostatic equivalent, rises from 146 to 175 when these values are determined by equations (1) and (2), and when V for adrenalectomized muscle is calculated on the assumption that a similar number of fibres are associated with the same dry weight of tissue. There is thus a fall in the total number of those characteristic molecules which the cell retains and which do not diffuse through its membrane. This is accompanied by a rise in the sum of their negative charges.

Computing η values in muscle from the direct analyses and omitting anserine, there appears a fall of about 9–10 mmol./kg. of fibre water due to the fall in total hexose esters and Na as against the small gains of phosphocreatine and magnesium. The remaining 17 mmol. would remain unaccounted for by the direct analyses.

The increase of ϵ, i.e. the increase in the negative charges on the non-diffusible molecules, must occur to accommodate the increase of K beyond what is accounted for by the decrease in Na.

The sodium change. Clearly any accurate assessment should be based on the same number of fibres, and using the dry weights, the same number is contained in 1·03 kg. of adrenalectomized muscle as in 1 kg. normal muscle. Now in 1 kg. normal muscle there are 27·1 mmol./Na, and in 1·03 kg. adrenalectomized muscle there are 20·3 mmol./Na. In the normal muscle s or the interspace is approximately 0·107 l. containing $0{\cdot}107 \times 138$ mmol. = 14·8 mmol.; hence $27{\cdot}1 - 14{\cdot}8$, or 12·3 mmol., are associated with the fibres. A similar calculation gives $20{\cdot}3 - (0{\cdot}087 \times 1{\cdot}03 \times 128) = 6{\cdot}9$ mmol. associated with the fibres in the adrenalectomized muscle. There is thus a fall of $12{\cdot}3 - 6{\cdot}9 = 5{\cdot}4$ mmol. It is obvious that the evidence for this change really rests on the somewhat uncertain ground that the same number of fibres are associated with the same total dry weight of muscle, but as it stands it amounts to a value of about 4 % of the external Na. There is at the same time a gain of K which, disallowing for the relatively very small interspace K, amounts to $(118{\cdot}5 \times 1{\cdot}03) - 101 = 21$ mmol./kg. There is thus no correspondence between the K gain and the Na loss.

The loss of Na raises the question as to whether it is in fact extruded from within the fibre. The authors consider it more reasonable to suppose that it represents a small Na loss from an essentially extracellular source, which may be provisionally regarded as the sarcolemma.

The hexose esters. Of much interest are the changes in the concentrations of glucose-1-phosphate, glucose-6-phosphate and fructose-6-phosphate. Such changes are relatively very large. Glucose-1-phosphate is increased to 134 % of the control value, and the other two decreased to 32 % and 37 %. We consider it preferable to defer an explanation of such changes to a later communication, with fuller studies of the carbohydrate changes, and further controls. At present, attention may be called to the fact that the effect on the glucose-1-phosphate appears to relate to the findings of Schumann (1940) confirmed by Verzár & Montigel (1942) in which it was shown that phosphorylase action was inhibited in excised muscle from adrenalectomized rats as compared with similar muscle from normal rats.

SUMMARY

1. Skeletal muscle of albino rats (almost all of Wistar strain) was analyzed for various constituents 3–6 days after adrenalectomy, and compared with similar analyses on control rats, maintained on the same diet (containing low Na). The substances determined were K, Na, Mg, Cl, total P, acid-soluble P, phosphocreatine, adenosinetriphosphate, total hexose-monophosphate, glucose-6-phosphate, glucose-1-phosphate, fructose-6-phosphate, fructose-1:6-diphosphate, phosphoglyceric acid, phosphopyruvic acid, carnosine, anserine, water content. An increase after adrenalectomy was shown for K, Mg, phosphocreatine, glucose-1-phosphate. A decrease was shown for Na, glucose-6-phosphate, fructose-1:6-diphosphate.

2. Plasma constituents for the same rats, or rats under similar conditions were also determined. The substances or factors investigated were K, Na, Ca, Mg, pH, Cl, HCO_3^-, inorganic P, total P, urea, total molecular concentration, water content. Following adrenalectomy there was an increase of K, Mg, urea, total phosphate and inorganic phosphate. A decrease was shown for Na, Cl, HCO_3^-, Ca and the pH being probably decreased.

3. The η value, or total non-diffusible substances (reckoned as mmol./l. of 'fibre water'; Boyle & Conway, 1941) falls from 147 to 120 after adrenalectomy; the ϵ value (or mmol. alkali/l. of 'fibre water' required to satisfy the surplus of negative charges on η) rises from 146 to 175. Somewhat less than half the fall in η is accounted for by the direct analyses of the muscle constituents.

Our thanks are due to the Irish Medical Research Council for a grant to one of us (D. H.).

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The Nucleoprotein Content of Fibroblasts Growing *in vitro*

3. THE USE OF DEFATTED MEDIA

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(*Received* 1 *May* 1946)

It is generally accepted that lipid materials are inhibitory to the growth of tissues *in vitro* (cf. Baker & Carrel, 1925*a*, *b*; Mayer, 1936). We have already shown that the removal of fat from sheep embryo extracts increases the growth-promoting power of such extracts for tissue cultures of chick heart fibroblasts (Davidson & Waymouth, 1945). It has therefore seemed to us desirable that the effect on the growth of cultures of removal of the lipids from the embryo extract of the plasma coagulum or both, should be further investigated. In the course of this work we have prepared fat-free fowl plasma in the form of a sterile powder which can be stored indefinitely (Waymouth & Davidson, 1945). The use of a large batch of such plasma powder enables a long series of cultures to be set up in the same medium and avoids the necessity for the maintenance of a stock of liquid plasma which must be renewed frequently and which may vary in composition from batch to batch.

The use of defatted plasma has also enabled further investigations to be made on a factor in pancreatin which profoundly influences the morphology of fibroblast cultures *in vitro* (Davidson & Waymouth, 1944*a*). This factor appears to be identical with, or related to, the enzyme lecithinase A which hydrolyzes lecithin to lysolecithin. To examine the effect of this enzyme on growing tissue defatted media are obviously desirable.

This paper deals with the phospholipin content of embryonic tissues and of embryo tissue extracts before and after defatting, with the preparation of defatted fowl plasma and its chemical composition, and with the use of these defatted media in tissue culture technique.

METHODS AND MATERIALS

Tissue culture technique

(*a*) *General.* Cultures were grown by the roller tube technique of Willmer (1942) which we have previously employed (Davidson & Waymouth, 1943, 1945). Each roller tube contained 12 pieces of tissue in two rows of 6. As in earlier work all the cultures were made from fresh explants from the 10-day chick embryo heart and consisted therefore to a large extent of the cells which are usually termed 'fibroblasts' in tissue culture work (Mayer, 1939) but which have been more precisely defined as 'mechanocytes' by Willmer (1945). The amount of tissue present was determined by making estimations of nucleoprotein phosphorus (NPP) (Davidson & Waymouth, 1943).

(*b*) *Materials.* Lecithin was prepared from egg yolk by the method of Macfarlane & Knight (1941), and dissolved in ethanol. Insoluble material was centrifuged off and an emulsion prepared by adding 2 ml. of the ethanolic solution to 25 ml. of boiling 0·9 % NaCl (Ponder, 1942). The solution was heated for 10 min. in a boiling water-bath to sterilize it and to drive off the ethanol. The concentration of this emulsion was estimated from the P content and suitable dilutions made with Tyrode solution.

Two samples of lysolecithin, of fractions 2 and 7 of King & Dolan (1933), were kindly presented by Prof. E. J. King. 5 mg. portions were dissolved in chloroform, reprecipitated with acetone and dissolved in 1·5 ml. of 0·9 % NaCl. The solutions were sterilized by heating in a boiling water-bath at 100° for 10 min., cooled and diluted with Tyrode solution.

The pancreatin preparations were prepared according to the method previously described (Davidson & Waymouth, 1944*a*). They were dissolved in water, sterilized by heating to 100° for 10 min., cooled, and treated with an equal volume of double strength Tyrode solution. Similar preparations were also heated in alkaline solution (pH 8–10) in order to destroy such resistant enzymes as lecithinase A and

ribonuclease. The solutions were then neutralized and treated as above.

Preparations of the heat stable enzyme lecithinase A were obtained from two sources. (i) A sample of Russell's viper venom provided by Boots Pure Drug Co. Ltd. was dissolved in water and sterilized by heating to 100° for 10 min. The denatured protein which appeared during the heating was centrifuged down and discarded. The supernatant fluid was treated with an equal volume of double strength Tyrode solution before use. (ii) From the dry venom of the rattlesnake, *Crotalus t. terrificus*, Slotta & Fraenkel-Conrat (1938*a*, *b*) have isolated the neurotoxic principle as a crystalline protein which they have named 'crotoxin'. This material has powerful lecithinase A activity. A sample of pure 'crotoxin' was kindly put at our disposal by Dr Fraenkel-Conrat who stated that it had been proved to be homogeneous when examined in the ultracentrifuge and in the diffusion apparatus (Gralen & Svedberg, 1938) and when subjected to electrophoresis experiments (Li & Fraenkel-Conrat, 1942). It was dissolved in water and treated in the same way as the Russell's viper venom.

Embryonic tissues and extracts

(*a*) In the preparation of '50%' embryo extract the embryo pulp was mixed with an equal volume of Tyrode solution and was either centrifuged after standing for a short time or was frozen and thawed before centrifuging. Extracts prepared by the latter method contained haemoglobin from the ruptured red blood cells.

(*b*) The embryo extract was defatted as previously described (Davidson & Waymouth, 1945) by freezing with ether according to the method used by McFarlane (1942) for plasma. In the case of chick embryo extract the whole process was carried out aseptically using sterile glassware.

(*c*) The phospholipin-P contents of embryo extract were determined by one of two methods. (i) A 10 ml. sample of embryo extract was added to about 50 ml. ethanol : chloroform (3 : 1), the mixture warmed on a hotplate and filtered. The residue was re-extracted and the combined filtrates made up to a total volume of 100 ml. 10 ml. of the filtrate were taken to dryness in an open dish on a water-bath, the residue extracted with pure chloroform, the extract filtered, and total phosphorus estimated in the chloroform filtrate by the method of Allen (1940). (ii) Embryo extract (2 ml.) was treated with 2 ml. of 20% (w/v) trichloroacetic acid, the residue washed twice with 10% (w/v) trichloroacetic acid, and extracted once with 5 ml. 80% (v/v) ethanol, once with 5 ml. absolute ethanol, three times with ethanol-ether (3 : 1) and once with ether. The ethanol and ether extracts were combined and taken to dryness. Phosphorus was estimated in the extract by the method of Allen (1940).

(*d*) The phospholipin-P content of individual organs and tissues of the 9–10-week sheep embryo and of the adult sheep was also determined. Weighed portions (about 1 g.) of fresh tissue were finely minced and extracted with warm ethanol : chloroform (3 : 1) to a total volume of 100 ml. filtered extract. 10 or 20 ml. of the filtrate according to the amount of phospholipin in the tissue were evaporated to dryness and the residue extracted with chloroform. The chloroform extract was filtered and its phosphorus content determined by the method of Allen (1940).

Fowl plasma

(*a*) *Preparation.* For the preparation of defatted plasma advantage was taken of the observation of Hardy & Gardiner (1910) and of Hewitt (1927) that lipins can be removed from protein-containing solutions without protein denaturation by treatment with ethanol-ether mixture at temperatures below −10°, followed by extraction with ether.

For the present purpose, all manipulations were carried out aseptically and sterile Pyrex glassware was used throughout.

The plasma was collected from the carotid arteries of brown Leghorn cockerels and was stored in sterile waxed test-tubes on ice.

A 500 ml. sterile Pyrex conical flask containing ethanol-ether (7 : 3) mixture (6 vol. of mixture to 1 vol. of plasma) was cooled in a freezing mixture until the contents were below −15°. The plasma was then added slowly from a sterile Pyrex graduated pipette while the flask was gently agitated to ensure thorough mixing. The temperature was not allowed to rise above −5°. The flask surrounded by the freezing mixture was set aside in the cold room for 2 hr. The protein precipitate was then centrifuged down in sterile 50 ml. centrifuge tubes covered with sterile caps, and washed on the centrifuge twice with cold ethanol-ether mixture and three times with cold ether. The ethanol-ether mixture used for washing was kept in a vessel immersed in a freezing mixture and the centrifuge tubes and buckets were chilled in a freezing mixture between runs. The precipitate was then transferred with the aid of a sterile spatula and a little ether to a sterile Soxhlet thimble. Extraction with ether in a sterile Soxhlet apparatus was carried out for two periods of 2 hr., a small amount of sodium being placed in the flasks. The thimble was then drained as nearly free from ether as possible and was transferred aseptically to a sterile glass vessel. The last traces of ether were removed in a vacuum desiccator and the dried plasma proteins were then powdered with a sterile spatula and transferred to sterile tared test-tubes (120–160 mg. per tube) for storage. The yield was about 30 mg. powder per ml. of plasma.

For reconstitution, sterile Tyrode solution was added in the proportion of 1 ml. to 30 mg. powder and the mixture was stirred with a sterile glass rod. It dissolved easily and completely yielding a clear colourless solution which was alkaline (about pH 8) and required the addition of a few drops of sterile 0·02 N-HCl to bring the pH to 7·4 (phenol red).

The reconstituted plasma contained sufficient prothrombin for clotting to occur readily when tissue fragments were added to it.

(*b*) *Analysis of defatted plasma.* Samples of defatted plasma reconstituted in water were compared with corresponding samples of the whole plasma. Total N was determined by the micro-Kjeldahl technique. Chloride was determined by the usual Volhard procedure and calcium by the method of Kramer & Tisdall (1921).

The phosphorus fractions were determined in 4 ml. of plasma which were treated with 4 ml. 10% (w/v) trichloroacetic acid. The precipitated proteins were centrifuged down and washed twice with 5% trichloroacetic acid. In the combined supernatant and washings inorganic-P and total acid-soluble-P were determined by the method of

Allen (1940). The protein precipitate was extracted with ethanol-ether or ethanol-chloroform and the extract used for the determination of lipid-P. The protein residue was ashed for the determination of residual P.

RESULTS

The phospholipin content of embryo tissues and extracts

The results of the estimations of phospholipin-P in the tissues of the embryo and adult sheep are shown in Table 1. On a fresh tissue basis the embryonic tissues have a much lower phospholipin-P concentration than the corresponding adult tissues, the difference being particularly marked in the case of liver and brain. The water content of 9–10-week sheep embryo tissues has already been recorded, and has been shown to be much higher for embryonic than for adult tissues (Davidson & Waymouth, 1944*b*). If the phospholipin-P content of the sheep tissues is calculated on a dry weight basis, it is again found that, except in the case of muscle and heart, the adult tissues are richer in phospholipin than those of the embryo, the difference still being most marked for liver and brain.

Table 1. *Phospholipin-P in sheep tissues*

	mg./100 g. fresh tissue								Mean values for Fresh tissue	Mean values for Dry tissue
Embryo										
Liver	56·3	53·0	64·4	46·4	64·9	—	—	—	57·0	334
Heart	42·8	45·6	44·2	48·1	48·8	—	—	—	45·9	353
Muscle	29·9	27·1	29·2	30·6	28·0	—	—	—	29·0	230
Brain	62·2	59·8	53·8	58·6	60·7	—	—	—	59·0	311
Kidney	44·6	48·8	42·9	43·6	43·5	—	—	—	44·7	409
Lung	40·8	31·3	27·9	22·8	28·1	25·5	—	—	29·4	267
Adult										
Liver	156·5	156·5	168·4	178·0	159·7	157·0	163·6	164·3	163·0	538
Heart	71·6	71·5	68·9	65·2	67·6	69·2	—	—	69·0	309
Muscle	46·7	39·5	44·25	45·9	30·1	27·8	—	—	39·1	123
Brain	166·6	192·9	161·0	147·8	164·2	157·4	—	—	165·0	793
Kidney	68·1	68·0	104·1	105·7	100·2	91·6	90·0	95·0	90·3	539
Lung	84·8	91·0	77·0	87·8	93·2	73·5	—	—	84·6	403

Since the phospholipin content of embryonic tissues is low, it is not surprising to find that the phospholipin-P concentration in embryo extracts is not high (Table 2). In the preparation of samples 1, 2 and 3 (Table 2) extracts were made both by simple extraction of the embryo pulp with Tyrode solution and by extraction after freezing and thawing. In all cases the extracts prepared by direct extraction had a slightly higher phospholipin content than those made after freezing.

Samples 4–8 (Table 2) were defatted by freezing with ether. This process removes a considerable amount, but not all, of the phospholipin-P and significantly reduces the nitrogen content. The proteins of chick embryonic tissues appear to be easily denatured and during the defatting process a certain amount of protein is removed on centrifuging. No similar protein loss occurs when plasma or serum is defatted by the same procedure, and the loss is much smaller during the defatting of sheep embryo extract.

Embryo extract contains a small amount of residual P probably derived from ribonucleic acid which is known to be present in embryonic extracts (Davidson & Waymouth, 1944*b*).

Table 2. *Composition of chick embryo extract*

Sample	mg./100 ml. Total N	Total P	Phospholipin-P	Residual-P
1 *a*	49·0	18·25	2·42	—
b Frozen-thawed	64·4	16·75	1·38	—
2 *a*	65·3	41·0	1·95	—
b Frozen-thawed	76·7	42·5	1·90	—
3 *a*	53·5	12·4	1·31	—
b Frozen-thawed	50·4	12·1	0·90	—
4 *a* Whole extract	154·0	—	2·49	—
b Defatted extract	88·5	—	0·44	—
5 *a* Whole extract	109·0	—	1·68	—
b Defatted extract	65·0	—	0·08	—
6 *a* Whole extract	96·0	—	2·28	—
b Defatted extract	47·0	—	0·51	—
7 *a* Whole extract	73·8	—	1·89	0·84
b Defatted extract	56·0	—	0·85	0·01
8 *a* Whole extract	76·0	17·2	2·24	1·14
b Defatted extract	48·0	10·3	0·61	0·37

The composition of fowl plasma

This is shown in Table 3. The analytical figures for five samples are recorded, each reconstituted defatted sample being compared with the corresponding whole plasma from which it was made. In

Table 3. *Composition of blood plasma of brown Leghorn cockerels*

	mg./100 ml.							
Sample	Total N	Non-protein-N	Total acid-soluble-P	Inorganic-P	Lipid-P	Residual-P	Chloride (as NaCl)	Calcium
1 A	491	—	2·70	2·45	3·54	0·26	590	—
B	352	—	1·98	1·82	0·34	0·07	37·5	—
2 A	581	—	3·28	3·13	3·63	0·20	683·5	—
B	424	—	2·20	1·99	—	—	14	—
3 A	357	—	3·90	3·30	3·06	0·24	660	8·7
B	265	—	1·90	1·75	0·12	0·08	20	—
4 A	449	27·4	4·52	4·25	2·21	0·10	700	10·7
B	317	12·6	2·40	1·95	0·17	0·04	10	9·1
5 A	599	37·8	3·80	3·60	4·00	0·44	755	10·4
B	352	11·7	3·50	3·30	0·10	0·02	15	8·6

A = whole plasma. B = defatted plasma powder reconstituted with water.

each case the defatted plasma has a lower total nitrogen concentration than the whole plasma. Although a small part of this fall can be accounted for by manipulative losses, the major part appears to be due to a loss of albumin, since the albumin-globulin ratio is much lower in the defatted than in the whole plasma. The defatted plasma still contains an appreciable amount of non-protein nitrogen.

The lipid-P content of whole cockerel plasma is not high and, as might be expected, the amount is greatly reduced in the defatted material. After extraction of acid-soluble-P and lipid-P from whole plasma, a small but fairly constant amount of P still remains. In the defatted plasma the amount of residual-P is negligible.

Of the inorganic constituents of plasma, chloride is present in only small amounts in the defatted material and in no case is more than 7% of the chloride of the plasma found in the extracted material. On the other hand up to 92% of the acid-soluble-P of the untreated plasma and of the inorganic-P which forms the greater part of this fraction in the plasma of cockerels, is retained in the reconstituted solution. A large proportion of the calcium is also retained in the defatted material.

The growth of cultures in defatted plasma

The nucleoprotein phosphorus (NPP) content of cultures grown in whole plasma and in defatted plasma is shown in Fig. 1. In confirmation of our previous observations (Davidson & Waymouth, 1943) it is found that cultures maintained in Tyrode solution alone show a marked fall in NPP (test 190, curve *D*). In its initial stages this fall is not prevented by the presence of embryo extract (curve *B*) but can be diminished to some extent in the presence of embryo extract of high concentration ($N = 90$)* (curve *A*). With moderate concentrations of embryo extract ($N = 30$) the NPP at the end of 4 days is much higher than in cultures grown in Tyrode solution alone but is still slightly lower than

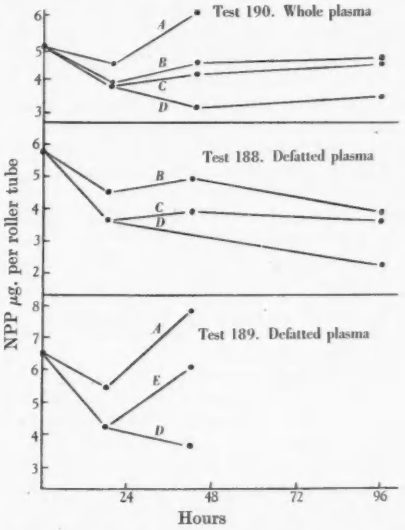

Fig. 1. Effect of chick embryo extract on the nucleoprotein phosphorus (NPP) content of cultures grown in whole plasma and in defatted plasma with and without embryo extract. Each roller tube contained 12 pieces of tissue and the cultures were set up as fresh explants at 0 hr. *A*, embryo extract ($N = 90$) present from time of setting up. *B*, embryo extract ($N = 30$) present from time of setting up. *C*, embryo extract ($N = 30$) added after 16 hr. in Tyrode solution. *D*, Tyrode solution alone as fluid phase. *E*, embryo extract ($N = 90$) added after 16 hr. in Tyrode solution.

when the cultures were first set up and the final figure is the same when the embryo extract is added after 18 hr. as when it is present from the

* This convention is employed as before (Davidson & Waymouth, 1945) to indicate the concentration of total N (as mg./100 ml.) in the fluid added to the roller tubes.

time of setting up (test 190, curves *B* and *C*). With high concentrations of embryo extract ($N=90$) a very sharp rise in NPP takes place during the second day and the figure after 2 days is much higher than at the time of setting up (test 190, curve *A*).

When cultures are grown in defatted plasma (Fig. 1, tests 188 and 189) with Tyrode solution alone as fluid phase (curve *D*) the fall in NPP in the course of 4 days is much greater than is the case with similar cultures grown in whole plasma, and the fall is much sharper in its initial stages. This sudden decrease in NPP in the course of the first 18 hr. can be abolished by the addition of peptone ($N=25$) and can be reduced by the addition of boiled kidney extract (Fischer & Astrup, 1942) ($N=25$), of malt extract prepared by the method of Astrup & Fischer (1945) ($N=50$), and of pancreatin preparations ($N=25$), but is not affected by lecithin ($N=5$) or choline ($N=4$).

In the presence of embryo extract the NPP of the cultures still shows a fall in the course of the first 18 hr. (test 188, curve *B*; test 189, curve *A*) although the fall is not so great as with Tyrode solution alone. With high concentrations of embryo extract ($N=90$) (test 189) a sharp rise in NPP follows the initial fall, and if the embryo extract has been present from the beginning (curve *A*), the figure for the NPP at the end of 2 days is much higher than at the time of setting up. The use of defatted plasma is therefore compatible with good growth of the explants as measured by increase in NPP. Comparison of curves *A* and *E* (Fig. 1, test 189) shows that the rise in NPP during the course of the second day is almost the same when the embryo extract ($N=90$) is added after 18 hr. as when it has been present from the start, although the final figure is of course much lower.

With low concentrations of embryo extract ($N=30$) (test 188, curves *B* and *C*) a slight rise in NPP occurs during the second day and this rise is almost of the same magnitude when the embryo extract is added after 18 hr. as when it has been present from the time of setting up, although again the absolute figure is higher in the latter case. During the third and fourth days the NPP falls again and curves *B* and *C* tend to converge.

Comparison of curves *B* and *C* in tests 188 and 190 (Fig. 1) shows that for $N=30$ whole plasma is more efficient than defatted plasma in maintaining the NPP levels of the cultures and the final values reached after 4 days are similar when the embryo juice is added after 18 hr. and when it is present from the start.

Fig. 2 shows a comparison of whole plasma with defatted plasma in maintaining the growth of cultures in whole and defatted extract. In both cases the test substances were added after the cultures had been maintained for 18 hr. with Tyrode solution alone as fluid phase. In both cases the NPP falls continuously when Tyrode solution alone is the test material (tests 192 and 193, curve *D*) and the fall is more pronounced with defatted plasma than with whole plasma.

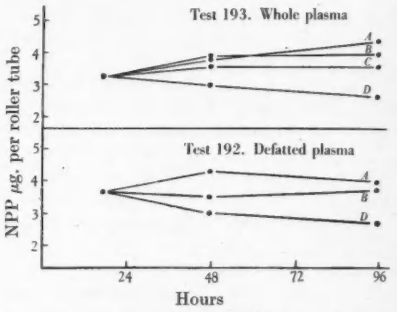

Fig. 2. Effect of whole and defatted chick embryo extract on the nucleoprotein phosphorus (NPP) content of cultures grown in whole plasma and defatted plasma. The cultures were maintained in Tyrode solution alone for 18 hr. before the test substances were added. *A*, test substance, whole embryo extract ($N=46$). *B*, test substance, embryo extract defatted by freezing with ether ($N=46$). *C*, test substance, defatted protein fraction of embryo extract ($N=46$). *D*, Tyrode solution alone.

Curves *A* and *B* in Fig. 2 show a comparison of whole extract with defatted extract. When whole plasma is employed (test 193), in contrast to the results obtained with sheep embryo extract (Davidson & Waymouth, 1945), whole chick extract is slightly more effective than defatted extract in causing a rise in NPP and the figures steadily increase during the 3 days of the test period.

With defatted plasma (test 192) the use of whole extract (curve *A*) is followed by a rise and then a fall in NPP. This is in accordance with the results obtained in Fig. 1, test 188, curve *C*. When defatted extract is employed (test 192, curve *B*) the NPP remains almost constant over the test period. The fall in NPP which occurs with Tyrode solution alone is prevented but no rise takes place. The difference between whole extract and defatted extract is thus more marked with defatted plasma than with whole plasma and defatted chick embryo extract does not appear to be able to cause an increase in NPP in cultures in defatted plasma.

In Fig. 2, test 193, curve *C*, the test medium was a solution of chick embryo proteins defatted by the method employed in preparing the defatted plasma powder. This defatting procedure caused a certain amount of protein denaturation and the soluble proteins in the final product did not maintain the cultures so well as did embryo extract defatted by freezing with ether.

The effects of lecithinase A and lysolecithin

Previous work has shown (Davidson & Waymouth, 1944*a*) that pancreatin extracts are able to increase the NPP content of tissue cultures and also to cause morphological changes resulting in the production of a dense culture with a sharply demarcated edge. The latter changes are thought to be due to the presence of the enzyme lecithinase A. We have therefore tested the effects on cultures growing in whole and defatted plasma of pancreatin extracts before and after heating in alkaline solution to inactivate the enzyme. Attempts were also made to restore the activity which is abolished by heating in alkaline solution, by the addition of Russell's viper venom and of pure crotoxin. Since it appears probable that lecithinase A exercises its effect by acting on the phospholipin either of the medium or of the cells (e.g. by producing lysolecithin from lecithin) it was thought desirable to test the action of the enzyme on cultures growing in a wholly defatted medium with and without the addition of lecithin. The effect of lysolecithin was also examined. Some of the results are shown in Table 4.

When whole plasma is employed (Table 4, tests 198 and 201) the addition of pancreatin extract to a medium containing embryo extract causes a higher rise in NPP than is produced by a moderate or low concentration of embryo extract alone. A pancreatin preparation which has been heated in alkaline solution is rather less effective in this respect. The addition of lecithinase A or lysolecithin to such a heated preparation results in the appearance of the characteristic morphological changes without however having any marked effect on the NPP. Higher concentrations of lecithinase or lysolecithin than those shown in Table 1 were toxic to the cells and resulted in a fall in NPP in the cultures.

In a medium containing defatted plasma and defatted embryo extract, the addition of pancreatin

Table 4. *The effect on the nucleoprotein phosphorus (NPP) content of tissue cultures of combinations of whole or defatted chick embryo extract (EE) with lecithinase A or associated substances*

(Each roller tube contained 12 pieces of tissue. Cultures were grown for 48 hr. in Tyrode solution alone and for a further 48 hr. in the test media.)

Test no.	Plasma	Test substance	Nitrogen concentration (mg./100 ml.)	Change in NPP of cultures (μg./roller tube)	Morphological change
198	Whole	Whole EE	10	+0·88	
		„ „ +pancreatin extract	10+50	+2·56	
		„ „ +pancreatin extract (heated)	10+50	+1·91	
		„ „ +pancreatin extract (heated)+crotoxin	10+50+0·07	+2·04	+ + +
		„ „ +pancreatin extract (heated)+lysolecithin (50 mg. %)	10+50	+1·61	+ +
		„ „ +pancreatin extract (heated)+Russell's viper venom	10+50+0·07	+1·36	+ +
201	Whole	Whole EE	38	+2·01	
		„ „ +pancreatin extract	38+50	+2·52	+
		„ „ +pancreatin extract (heated)	38+50	+1·94	
		„ „ +pancreatin extract (heated)+crotoxin	38+50+0·07	+2·16	+ +
201	Whole	Whole EE+pancreatin extract (heated)+lysolecithin (50 mg. %)	38+50	+2·13	(+)
		„ „ +pancreatin extract (heated)+Russell's viper venom	38+50+0·07	+2·61	+ +
200	Defatted	Defatted EE	40	+1·53	
		„ „ +pancreatin extract	40+50	+0·66	
		„ „ +pancreatin extract (heated)	40+50	+1·71	
		„ „ +pancreatin extract (heated)+crotoxin	40+50+0·07	+0·61	
		„ „ +pancreatin extract (heated)+lysolecithin (50 mg. %)	40+50	+0·69	
		„ „ +pancreatin extract (heated)+Russell's viper venom	40+50+0·07	+1·39	
207	Defatted	Whole EE	20	+0·52	
		„ „ +lecithin	20+1	+0·53	
		„ „ +lecithin	20+5	+1·05	
		„ „ +crotoxin	20+0·07	+0·47	+
		Defatted EE	20	+0·72	
		„ „ +lecithin	20+1	+0·60	
		„ „ +crotoxin	20+0·07	+0·64	+
		„ „ +crotoxin+lecithin	20+0·07+1	+0·40	+

extract does not further increase the NPP of the cultures, and morphological changes visible to the naked eye are not seen after the addition of lecithinase A or lysolecithin (Table 4, test 200).

The effect of defatted embryo extract on the NPP of cultures in defatted plasma as measured by the difference between the NPP in the test medium and the NPP in Tyrode solution alone at the end of the growth period, was of the same order as that produced by whole extract (Table 4, test 207). A low concentration of lecithin had no effect in either case and a higher concentration of lecithin had none of the inhibitory effects usually attributed to phospholipins. Crotoxin produced morphological changes when added to these media even in the case of the wholly defatted media though in defatted plasma the effect was seen only on microscopical examination.

DISCUSSION

The phospholipin contents of the tissues in the embryo sheep are in general lower than in the adult. In this respect the sheep embryo resembles the rabbit embryo in which Boyd (1935) found a rise in phospholipin-P during intra-uterine growth. In the pig embryo on the other hand, though the lipin-protein ratio remains constant for a large part of the gestation period, the phospholipin content shows no increase (Gortner, 1945).

We have already observed (Davidson & Waymouth, 1945) that '50%' chick embryo extract has a much lower nitrogen concentration than '50%' sheep embryo extract. The phospholipin-P content of sheep extract is usually 1–2 mg. per 100 ml. and therefore does not differ greatly from that of chick extract.

Although numerous analyses of the blood of laying and non-laying hens have been published, there are few figures on record for the blood of the cockerel. Our figures for the phospholipin-P content of the plasma of the brown Leghorn cockerel are of the same order as those of Boyd & Clarke (1940) and are lower than those of Roepke & Hughes (1935). In the plasma of the laying hen the phospholipin-P content is very much higher. Our figures for inorganic phosphate, which forms a very large proportion of the total acid-soluble-P, are slightly lower than those of Benjamin & Hess (1933) (6·1) and of Roepke & Hughes (1935) (5·4) but are well within the range (1·2–5·6, mean 3·8) quoted by Mayrs (1923). Our figures for calcium agree well with those of Benjamin & Hess (1933) (10·7) and are slightly lower than those of Correll & Hughes (1933) (11·7), of Roepke & Hughes (1935) (11·2), and of Zondek & Marx (1939) (11·2).

The procedure of treating plasma with ethanol and ether at a low temperature removes nearly all the lipid-P. The residual-P is also greatly reduced. Of the inorganic constituents of the defatted plasma reconstituted in water, the chloride content is very low but calcium and inorganic phosphate are not much lower than in whole plasma. This may be due to the relative insolubility of these substances in the plasma-solvent mixture at the low temperature employed or it may be that at least part of the calcium and phosphorus is bound to protein in a loose type of combination which is disrupted by deproteinizing procedures (cf. Grollman, 1927). In this connexion it should be noted that Mayrs (1923) observed that the phosphates of cockerel serum were only partly ultrafilterable, while Greenberg, Larson, Pearson & Burmester (1936) found that the fowl differed from higher vertebrates in having part of its inorganic-P in non-diffusible form.

It is clear from Fig. 1 (test 189, curve *A*) that with a sufficiently high concentration of embryo extract good growth of tissue cultures, as measured by increase in NPP, is possible in defatted plasma. Comparison of tests 188 and 190, however, shows that, in general, cultures deteriorate more quickly in defatted than in whole plasma when the concentration of embryo extract is the same. It appears probable therefore that the plasma clot acts not merely as an inert mechanical support for the growing cells but also contributes factors to their nutritive requirements. Whether these factors are to be found in the lipid fraction or among the non-lipid materials removed in the defatting process is not certain.

Previous work on the effect of lipins on tissue cultures, e.g. by Mayer (1936), who used a mixture of brain lipins, has mostly been carried out with very complex mixtures and the inhibitory effects found have not been attributed to any particular fraction. Carminati (1932) found that a synthetic (distearyl) lecithin had a distinct growth-promoting effect on chick heart cultures. There is thus no evidence that the lipins removed from plasma are necessarily inhibitory.

We have previously found that tissue cultures in whole plasma grow better in defatted than in whole sheep embryo extract (Davidson & Waymouth, 1945). With chick embryo extract, the defatting process slightly reduces the growth-promoting power of the extract and this reduction is more marked when defatted plasma is employed. Cultures do not grow well in a medium consisting of defatted plasma and defatted embryo extract.

The proportion of protein lost in the defatting of chick extract (where the total protein content is relatively low) is much greater than with sheep extract. The difference between the effects of whole and defatted extract may therefore be due not only to the loss of lipin but to alteration in the relative proportions of some of the other constituents.

If the morphological changes produced by extracts of pancreatin are in fact due to the presence

of lecithinase A, it is probable that the enzyme exerts its effect by producing lysolipins from the phospholipins of the plasma, the embryo extract or of the cells themselves. The lysolipins, which produce their effects on erythrocytes and other cells by lowering the surface tension of the medium and of the cells (Bleyl, 1945) may act upon the cells of the culture to produce a change in shape similar to the prelytic swelling and loss of discoidal form of red cells observed by Ponder (1943). Preparations from Russell's viper venom, and also pure crotoxin, in suitable concentrations can act on cultures grown in whole plasma and embryo extract to produce morphological changes similar to those observed with pancreatin extracts. Lysolecithin has a similar effect.

The same concentration of lecithinase A produces very little change in morphology in cells in a defatted medium, and such change as occurs may be due to a direct effect of the enzyme on the phospholipins of the cells, or on the traces of phospholipins remaining in the plasma and embryo extract after treatment with ether.

SUMMARY

1. Estimations have been made of the phospholipin-P concentration in the tissues of the embryo and adult sheep. The phospholipin concentration is in general lower in the embryonic than in the corresponding adult tissue.

2. Cockerel plasma has been prepared in the form of a sterile defatted powder which dissolves freely in water and in Tyrode solution and retains its clotting power. Such defatted material is suitable for tissue culture work.

3. Tissue cultures consisting of fresh explants of the 10-day chick embryo heart can be maintained in a coagulum prepared from defatted plasma, and in the presence of a suitable concentration of embryo extract will show growth as measured by increase in NPP. Cultures deteriorate more quickly in defatted plasma than in whole plasma.

4. Defatted chick embryo extract is less effective than whole chick embryo extract in promoting the growth of cultures. The NPP of cultures grown in defatted plasma and defatted embryo extract is maintained, while in defatted plasma and Tyrode solution there is a marked drop.

5. Changes in the morphology are produced in the cells of cultures in media containing lecithinase A and these changes are probably due to the effect of this enzyme on the phospholipins of the medium and the cells. Lysolecithin produces a similar effect.

Our thanks are due to Miss M. M. Burchell for valuable technical assistance. Some of the experiments described in this paper were carried out in the University of Aberdeen in the spring and summer of 1945.

Note added 29 *June* 1946. Since this paper was written we have been able to consult a paper by J. L. Delsal (*Bull. Soc. Chim. Biol.* 1944, **26**, 282) who defatted human and horse serum by Hewitt's method and dissolved the protein powder in water. The reconstituted serum contained nearly all the inorganic-P present in the original material but only 10–20 % of the NaCl.

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The Availability of Histidine Derivatives for Growth

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(*Received* 8 *May* 1946)

The purpose of the experiments to be described in this paper was to investigate whether the rat can synthesize the glyoxaline ring of histidine, if certain possible precursors are provided in the diet. It was first shown by Rose & Cox (1924) that a diet in which protein was supplied in the form of a casein digest freed of histidine by silver precipitation could not support growth in young rats. This was later confirmed with synthetic diets (Rose, 1938). On the other hand Burroughs, Burroughs & Mitchell (1940) claimed that histidine is not necessary for the maintenance of nitrogen equilibrium in the adult rat, and Rose, Haines, Johnson & Warner (1943) found that a positive nitrogen balance could be maintained in man in the absence of dietary histidine. It appeared to us that these various and to some extent contradictory findings could be explained by assuming that the mammalian organism is able to synthesize histidine from other aliphatic substances, but that the formation of these precursors is slow and is the rate-determining step in the biosynthesis of histidine. If this is the case the addition of such possible precursors to a histidine-deficient diet should lead to a stimulation of growth.

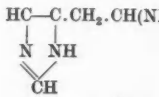
Little is known of the mechanism of the synthesis of iminazole compounds in plants, but by analogy with chemical methods it appeared possible that such a reaction could proceed from ornithine (I) through γ-hydroxy-ornithine to the keto-acid (III). The postulated introduction of a hydroxyl group into a position next to an amino group is a reaction which can presumably be performed by the organism, since both hydroxy-lysine and hydroxy-proline, which have this structure, are found in animal proteins and are not essential amino-acids; they must be presumed to be formed from lysine and proline by such a process of oxidation. The keto-acid (III) could then form histidine by any of the following routes: it could be oxidized to the dicarbonyl compound (IV) which could then condense with ammonia and formaldehyde to give histidine (V); such a reaction would be similar to the formation of 4(5)-methyl-glyoxaline from sugars (Windaus, 1907) which is assumed to involve the intermediate formation of methyl-glyoxal. The keto-acid (III) could also directly combine with formic acid and ammonia; for this there is no exact chemical analogy, but the Bamberger fission of iminazoles represents a reversal of such a reaction. Lastly, the acid (III) might form 2-thiol-histidine (VI) by condensation with thiocyanate, which is normally present in serum and saliva although in small amounts.

$H_2N(CH_2)_3.CH(NH_2)COOH$ (I)

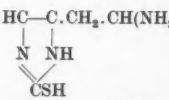
$H_2NCH_2.CHOH.CH_2.CH(NH_2).COOH$ (II)

$H_2N.CH_2.CO.CH_2.CH(NH_2).COOH$ (III)

$OHC.CO.CH_2.CH(NH_2).COOH$ (IV)



(V)



(VI)

It was expected that 2-thiol-histidine would easily be oxidized to histidine in the body. This paper describes experiments to test the ability of these two compounds, 2-thiol-histidine and $\alpha\delta$-diamino-γ-keto valeric acid to replace histidine in the diet. Opportunity was also taken to test the availability of α-*N*-acetyl and of α-*N*-benzoyl histidine for growth.

EXPERIMENTAL

Rats from one litter of black and white animals (National Institute for Medical Research strain) with weights of 46–51 g. were given the experimental diets. Weights were recorded every second day and food consumption was also measured,

Diet. The non-protein part of the basal diet was: cane sugar 40%, corn starch 41%, cod-liver oil in arachis oil (1 : 10) 14%, and salt mixture (*U.S. Pharmacopoeia*, **2**, no. 2) 5%. The histidine-free amino-acid mixture had the following composition: glycine 6·0, *dl*-alanine 12·0, *dl*-

valine 28·0, *l*-leucine 20·0, *dl*-isoleucine 20·0, *dl*-serine 6·0, *dl*-threonine 16·0, *l*-proline 12·0, *l*-hydroxy-proline 4·0, *l*-cystine 5·0, *dl*-methionine 10·0, *l*-glutamic acid 44·0, *l*-aspartic acid 8·0, *l*-arginine hydrochloride 13·0, *l*-lysine hydrochloride 14·0, *l*-phenylalanine 8·0, *l*-tyrosine 13·0, *l*-tryptophane 4·0, and $NaHCO_3$ 16·0. Most of these amino-acids were prepared in this laboratory, but some were obtained from Merck and Company, Inc., Rahway, N.J., U.S.A. The purity of the amino-acids was checked by analysis, and where possible, by measurement of optical rotation. The amino-acid mixture was well mixed with any supplement to be tested and the whole ground up with the non-protein part of the diet and made into a stiff paste by the addition of a very small amount of water. Vitamin supplements were added in the same amounts as described in an earlier paper (Gillespie, Neuberger & Webster, 1945)

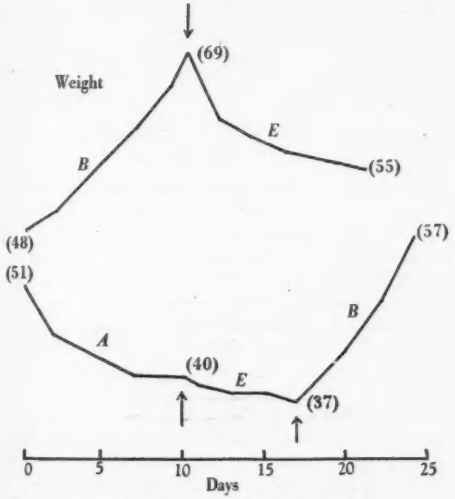


Fig. 1. The availability of 2-thiolhistidine for growth. The arrows indicate a change of diet. The weights of the animals at the beginning and end of each feeding period are shown in brackets. The diets fed to the animals were as follows: *A*, basal diet; *B*, basal diet + 35 mg. *l*-histidine/day; *E*, basal diet + 100 mg. 2-thiolhistidine/day.

except that the amount of choline chloride was increased to 12 mg./day. An amount of a specially prepared protein-free yeast extract (80 mg. (dry weight) equivalent to 0·5 g. of whole yeast) was also given. The intake of amino-acids was kept constant as far as possible, both during the deficiency and control periods, at 1·4 g./day, and variations in appetite were allowed for by adjustment of the intake of the non-protein part of the basal diet. On some days, however, the intake of amino-acids was slightly lower than 1·4 g., especially during the deficiency periods.

Preparation of compounds. *dl*-2-Thiolhistidine and αδ-diamino-γ-keto-valeric acid hydrochloride were prepared by the methods of Harington & Overhoff (1933), whilst for α-*N*-acetyl-*l*-histidine and α-*N*-benzoyl-*l*-histidine the directions of Bergmann & Zervas (1928) and Gerngross (1920), respectively, were followed.

RESULTS

Changes of weight on the basal diet

With histidine absent from the diet there was a loss of weight, amounting on the average to 1 g./day, at least for the comparatively short periods under consideration. The daily food consumption during the periods of deficiency averaged 4·5–5 g. The animals appeared to be quite healthy and lively.

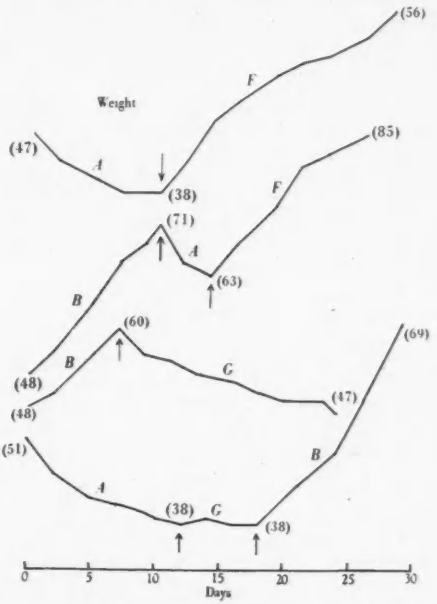


Fig. 2. The availability of *N*-acetyl and of *N*-benzoyl *l*-histidine for growth. The arrows indicate a change in diet. The weights of the animals at the beginning and end of each feeding period is shown in brackets. The diets fed to the animals were as follows: *A*, basal diet; *B*, basal diet + 35 mg. *l*-histidine/day; *F*, basal diet + 56 mg. *N*-acetyl-*l*-histidine/day; *G*, basal diet + 100 mg. *N*-benzoyl-*l*-histidine/day.

Changes of weight on the basal diet supplemented with histidine

An addition of 35 mg. *l*-histidine per day to the diet produces a considerable increase of body weight, the growth rate varying in different animals between 1·9–3·3 g./day, Figs. 1 and 2. It is not easy to compare the growth rates observed in experiments in which some amino-acids are given in the racemic form, with those found in which complete protein is used. If it is assumed, however, that the *d*-forms of the essential amino-acids used in these experiments are not available for growth, it can be calculated

that the amino-acid mixture consumed corresponds to a daily protein intake of 1·0 g. On this basis the average growth rate observed with a complete amino-acid mixture compared not unfavourably with results obtained with casein.

Addition of histidine to the basal diet produced an immediate and pronounced response both as regards change of body weight and increase in food consumption. Weight increased by 5–6 g. during the first 2 days after the addition of the amino-acid, and food consumption rose by about 100%. This quick and marked response facilitated greatly the testing of the ability of the various compounds to replace histidine in the diet.

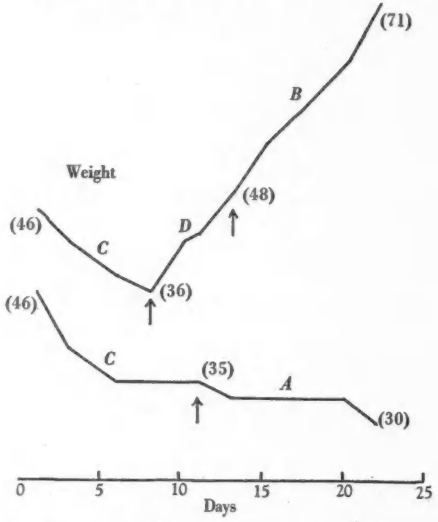


Fig. 3. The availability of αδ-diamino-γ-ketovaleric acid for growth. The arrows indicate a change of diet. The weights of the animals at the beginning and end of each feeding period are shown in brackets. The diets fed to the animals were as follows: *A*, basal diet; *B*, basal diet + 35 mg. *l*-histidine/day; *C*, basal diet + 100 mg. αδ-diamino-γ-ketovaleric acid/day; *D*, basal diet + 100 mg. αδ-diamino-γ-ketovaleric acid/day + 35 mg. *l*-histidine/day.

The availability of αδ-diamino-γ-ketovaleric acid for growth

The inability of this compound to replace histidine is clearly shown in Fig. 3. The average daily loss of weight in the two animals tested amounted to 1·1 and 1·25 g. respectively, and the daily food consumed averaged 4·1 and 4·5 g. respectively. The keto-acid was given as a hydrochloride in a daily dose of 100 mg., which corresponds to about 85 mg. of *dl*-histidine, or if we assume that only the *l*-form is available, to 43 mg. of *l*-histidine. The possibility of the substance being toxic can be excluded, since addition of histidine to a diet containing the keto-acid restored normal growth at once (Fig. 3). It is concluded therefore that αδ-diamino-γ-ketovaleric acid is not converted into histidine to any appreciable extent.

The availability of dl-2-*thiolhistidine for growth*

The results with this amino-acid were equally negative (Fig. 1). The daily dose given was 100 mg. which corresponds to about 83 mg. of *dl*- or to 42 mg. of *l*-histidine. Daily loss of weight in two experiments amounted to 0·4 and 1·5 g. respectively; the differences between these two values can be explained by the different initial weights of the two animals. Daily food consumption averaged 5·0 and 5·5 g. respectively. It is concluded that 2-thiolhistidine does not support growth of young rats kept on a diet free of histidine.

Availability of α-N-*acetyl and* α-N-*benzoyl*-l-*histidine for growth*

Fig. 2 shows clearly that whilst the acetyl compound replaces histidine, the benzoyl compound is inactive. The average daily weight gains of the two animals kept on a diet containing 50 mg. of acetylhistidine were 1·3 and 1·7 mg. respectively and the corresponding figures for average daily food consumption were 7·0 and 11·9 g. It follows that, at least at the level fed, the acetyl compound is as effective or nearly as effective as the amino-acid itself in supporting growth.

In the two experiments in which benzoylhistidine was given there was no increase of weight; in one experiment there was a loss of weight averaging 0·5 g./day, whilst in the other the body weight remained constant. The values for daily food consumption were similar to those found with the basal diet alone; they were 4·8 and 5·2 g. respectively. It is concluded that benzoyl-histidine cannot support growth on a histidine-deficient diet.

DISCUSSION

The results reported in this and two other papers (Glynn, Himsworth & Neuberger, 1945; Fuller, Neuberger & Webster, 1946) show that a diet supplying 1·3–1·4 g. of a mixture of amino-acids, some of which are racemic, can support growth in the rat at a rate of about 2 g./day for long periods. It has been possible to rear rats of initial weights of about 40 g. to full maturity on such diets and the final body weights observed after 100 days were between 230 and 250 g. This compares quite well with growth rates observed on diets containing 8% casein as a sole source of protein. At least at this comparatively low nitrogen intake amino-acid

mixtures are equal to whole protein and the *d*-isomers present in the diet have no apparent deleterious effect. At higher levels of intake, whole protein appears to be superior to amino-acid mixtures (unpublished observations); it is uncertain whether this difference is due to the presence of racemic forms in greater absolute concentrations in such nitrogen-rich diets or to the absence of specific growth factors, such as peptides (Woolley, 1945).

If histidine is left out of the amino-acid mixture there is an immediate and sustained loss of weight, and it is clear that histidine is required for the maintenance of body weight in the young rat. Similar findings have recently been reported by American workers (Albanese & Frankston, 1945; Maun, Cahill & Davis, 1946). These results suggest, but do not prove, that histidine cannot be synthesized at all by the rat. However, the fact that the loss of weight on the histidine-deficient diet found in these and other experiments (Fuller *et al.* 1946) is of the same order of magnitude as that found in deficiencies of essential amino-acids such as methionine, etc., renders very unlikely the assumption that histidine can be made by the rat from other constituents of the diet. The results reported in this paper show clearly that neither $\alpha\delta$-diamino-γ-ketovaleric acid nor 2-thiolhistidine can be converted to histidine by the rat. 2-Thiolhistidine is easily oxidized *in vitro* by ferric chloride to histidine; the observation that such an oxidation can apparently not take place *in vivo* is rather surprising. Ergothioneine does not replace histidine in the diet (Eagles & Cox, 1928), but this is not unexpected since other betaines cannot be converted into their parent amino-acids in the animal body (Jackson, 1929).

After the work described here had been completed a paper by Astwood, Bissell & Hughes (1945) reporting marked goitrogenic activity for 2-mercapto-iminazoles, came to our notice. Unfortunately, the thyroid glands of our animals had not been examined and we are therefore ignorant whether any changes in the thyroid may have occurred. The question, however, arises, whether the observed loss of weight, shown by the animals on the diet containing 2-thiolhistidine, could be ascribed to a goitrogenic or general toxic effect of the compound. Though this interpretation cannot be completely excluded, it appears unlikely. The sudden drop of weight which occurred as soon as histidine was replaced by the thiol derivative, and the immediate growth response on changing back to histidine, are most easily explained by assuming that we were dealing with a simple histidine deficiency. Moreover, the animals appeared to be quite healthy during the period when thiolhistidine was given.

The findings with respect to the two acyl derivatives bring histidine into line with most other amino-acids. In all cases so far tested the *N*-benzoyl derivatives do not support growth whilst the acetyl compounds excepting α-*N*-acetyl-lysine (Neuberger & Sanger, 1943) are fully active. The different behaviour of the α-acetyl derivatives of lysine and histidine may be attributed to the much greater basicity of the ϵ-amino-group of lysine as compared with that of the iminazole group of histidine. Thus at neutral pH the former exists mainly as a cation and may interfere with deacetylation by the appropriate enzyme, whilst the latter is mainly uncharged. It would be interesting to test this explanation by investigating the availability to birds of α-*N*-acetyl arginine which should resemble α-*N*-acetyl-lysine.

The findings reported by other workers and ourselves permit us to define the structural requirements of substances which are able to replace histidine in the diet, i.e. to be converted into this amino-acid by the organism. It appears that the glyoxaline ring must be present and not substituted in any other position than the 4 (5) position. Thus $\alpha\delta$-diamino-γ-ketovaleric acid, thiolhistidine and *l*-methylhistidine (Sakami & Wilson, 1944) are inactive. The alanine side chain, however, may be modified considerably without affecting activity. Thus the *d*-isomer (Conrad & Berg, 1936), β-iminazole α-hydroxy-propionic acid, the corresponding α-keto acid (Cox & Rose, 1926; Harrow & Sherwin, 1926), α-*N*-methylhistidine (Fishman & White, 1936) and the α-*N*-acetyl compound are all active. These derivatives can be converted into histidine by reactions known to occur with other compounds in the body. β-Iminazole-propionic acid and urocanic acid are, as might be expected, not convertible into histidine, and therefore not available for growth (Cox & Rose, 1926). α-*N*-benzoyl-histidine also belongs to this group.

SUMMARY

1. A diet which provided 1·3–1·4 g. of a mixture of 18 amino-acids, some of which were in the racemic form, produced in young rats a rate of growth which was similar to that found with a diet containing 8% casein. Omission of histidine led to loss of weight.

2. $\alpha\delta$-Diamino-γ-ketovaleric acid and 2-thiolhistidine did not support growth of rats kept on histidine-deficient diets. The inability of 2-thiolhistidine to replace histidine is considered not to be associated with the goitrogenic character of 2-mercapto-iminazoles and it is thought that neither substance can be converted to histidine by the rat.

3. Addition of α-*N*-acetylhistidine to a deficient

diet produced normal growth, whilst *N*-benzoylhistidine was inactive. The structural factors determining the ability of histidine derivatives to replace the amino-acid in the diet are considered.

We wish to acknowledge the assistance of the Rockefeller Foundation in obtaining certain of the amino-acids used, and of Glaxo Laboratories Ltd. for providing the yeast preparation.

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The Identification of Amino-acids Derived from Cystine in Chemically Modified Wool

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(*Received* 15 *May* 1946)

Consden, Gordon & Martin (1944) showed that all the naturally occurring amino-acids could readily be identified by means of partition chromatography on paper. Using the same methods it is shown in the present work that a number of amino-acids derived from cystine can also be identified as components of hydrolysates of wools which have been modified in various ways. Such hydrolysates have been examined for the following five amino-acids: cysteic acid, lanthionine, djenkolic acid, thiazolidine carboxylic acid and *S*-methyl cysteine. The production of these acids from cystine is represented schematically below:

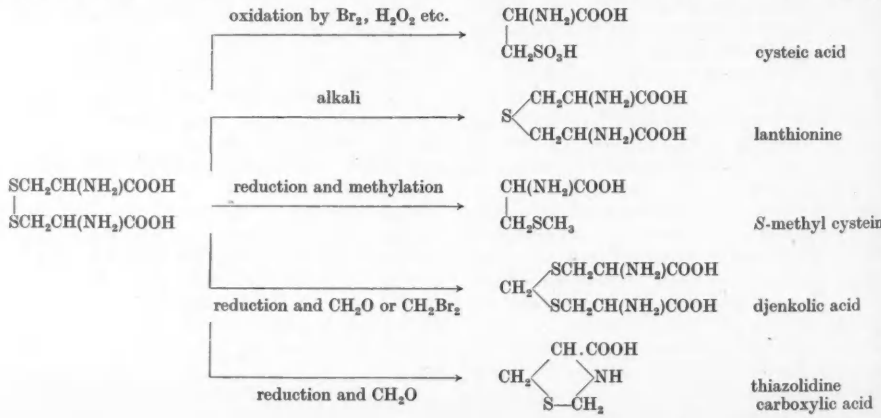


Table 1. *R_F values of some amino-acids derived from cystine on Whatman no. 4 paper**

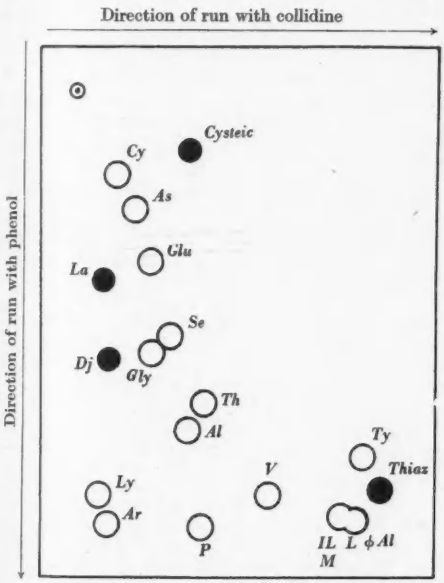
Solvent ... ... Addition	Phenol-cupron	Phenol-0·1% NH_3†	*S*-Collidine	Benzyl alcohol-cupron	Benzyl alcohol-HCN	*n*-Butanol-HCN	Colour with ninhydrin
Cysteic acid	0·14	0·11	0·34	0	0	0	Blue-purple‡
Lanthionine	0·22	0·30	0·12	0	0	0	Red-purple
Thiazolidine carboxylic acid	0·80	0·75	0·71	—	0·15	—	Yellow
Djenkolic acid	0·41	0·46	0·14	0·01	—	—	Purple
S-Methyl cysteine	0·75	—	0·45	0·11	—	0·16	Purple

* The R_F values of some of the common amino-acids are somewhat higher on Whatman no. 4 paper than on no. 1 paper.
† The percentage figure refers to the strength of the NH_3 solution present in the tray at the bottom of the chamber.
‡ Upper part pink in phenol.

Table 1 shows the R_F values of these amino-acids in various solvents.

Cysteic acid

Of the common amino-acids this is the slowest moving in phenol-NH_3 and hence a one-dimensional chromatogram suffices to reveal its presence (Pl. 5*a*). It does not appear to occur in that part of wool which in its natural state is close to the skin but occurs in small amounts in the tips of the fibres, where the wool has been exposed to weather and light (Pl. 6). It is of interest that a small amount of cysteic acid, but no cystine, was found to be present in a piece of bath sponge, which is usually prepared by allowing the living sponge to rot in the sun. When dry wool is very vigorously agitated in benzene, a small proportion of the cuticle cells (scales) becomes detached. These were found to contain a relatively large amount of cysteic acid and it is reasonable to suppose that these scales come almost exclusively from the tips of the wool. As would be expected, commercial wool material, which of course includes the fibre tips, showed a small amount of cysteic acid as a normal constituent.



Text-fig. 1. Key to Plate 6. *Al*, alanine; *Ar*, arginine; *As*, aspartic acid; *Cy*, cystine; *Dj*, djenkolic acid; *Glu*, glutamic acid; *Gly*, glycine; *IL*, *iso*leucine; *La*, lanthionine; *L*, leucine; *Ly*, lysine; *M*, methionine; *φAl*, phenylalanine; *P*, proline; *Se*, serine; *Thiaz*, thiazolidine carboxylic acid; *Th*, threonine; *Ty*, tyrosine; *V*, valine.

Lissizin (1928) isolated cysteic acid from human hair oxidized with permanganate, and many oxidizing agents readily convert cystine to cysteic acid (Friedmann, 1902; Andrews, 1933). It would therefore be expected that oxidizing agents would convert some of the cystine of wool to cysteic acid (Elliott, Nilssen & Speakman, 1938). During observations of ζ potentials of normal and chlorinated wool in 1940, Martin (unpublished) observed that the isoelectric point of the latter was at a pH value < 1. This can probably be explained only by the presence of strongly acidic groups, such as sulphonic acids. No direct demonstration of the presence of cysteic acid in wool has hitherto been published. We have been able to demonstrate the presence of increased amounts of cysteic acid in wool which has been given a non-shrink finish by dry or wet chlorination and in wool which has been bleached with H_2O_2. If dry-chlorinated wool is treated with papain at the appropriate pH (Phillips, Middlebrook & Higgins, 1941) that part of the fibre which has been acted upon by the chlorine is dissolved. As is to be expected, the cysteic acid content of such wool was found to be lower than that of the correspondingly chlorinated wool. An alkaline extract of chlorinated wool, when acidified (pH < 6), precipitates degraded protein material (Grieve, 1945). Two-dimensional chromatography shows that this material is devoid of cystine and very rich in cysteic acid.

Lanthionine

We have not been able to find any single solvent which would distinguish lanthionine from the other amino-acids of a wool hydrolysate. However, a two-dimensional chromatogram using *S*-collidine in one direction and phenol-coal gas-NH_3 in the other reveals its presence. Horn, Jones & Ringel (1941) first isolated lanthionine from alkali-treated wool and we also have been able to demonstrate the presence of this amino-acid in alkali-treated wool (cf. Cuthbertson & Phillips, 1945). A small amount of lanthionine has also been observed in tips of virgin wool (Pl. 6), and a larger amount in commercial flannel, probably due to alkaline scouring and subsequent heat treatment.

Djenkolic acid

The same two-dimensional chromatogram which is used to reveal lanthionine will also show djenkolic acid. We have confirmed the production of this amino-acid in reduced wool treated with methylene bromide (Harris, Mizell & Fourt, 1942) or with formaldehyde (Middlebrook & Phillips, 1946).

Thiazolidine carboxylic acid

This acid moves faster in collidine than any of the common amino-acids (Pl. 5*b*) and gives a yellow colour, with ninhydrin, which is slightly browner than that given by proline. Our findings on wools variously treated under different conditions with formaldehyde are given by Middlebrook & Phillips (1946).

S-*Methyl cysteine*

A strip, using *n*-butanol, shows the presence of this amino-acid as a spot in the gap between proline and valine (Pl. 5*c*). We have thus confirmed the formation of *S*-methyl cysteine in bisulphited wool treated with methyl sulphate (Blackburn, Consden & Phillips, 1944).

DISCUSSION

The identification of the foregoing amino-acids has been achieved by straightforward extension of methods previously employed. An interesting point, however, has arisen in the case of cysteic acid, which, when run in phenol without the addition of NH_3 shows a complex spot with ninhydrin, the upper portion being strong pink, and the lower part blue-purple. The presence of ammonia in the atmosphere reduces the amount of pink and in collidine a simple blue spot is observed. The phenomenon is essentially similar to that of the faster moving 'pink fronts', due to combination of copper, present in the paper, with all the other amino-acids. We have shown that the slow running pink spot behaves identically with the zinc salt of cysteic acid. In an attempt to isolate cysteic acid from a chlorinated wool hydrolysate using a paper-powder column, the whole of the cysteic acid was converted to the zinc salt. No other common amino-acid appears to form such a stable zinc complex.

SUMMARY

1. Methods are given for the identification, by partition chromatography on paper, of cysteic acid, lanthionine, djenkolic acid, thiazolidine carboxylic acid and *S*-methyl cysteine in protein hydrolysates.

2. Cysteic acid has been identified in samples of oxidized wool.

3. The presence of small amounts of lanthionine and cysteic acid has been demonstrated in the tips of virgin wool.

We thank Dr S. Blackburn and Mr W. R. Middlebrook for samples of variously treated wools. Thanks are also due to the Director and Council of the Wool Industries Research Association for permission to publish this work.

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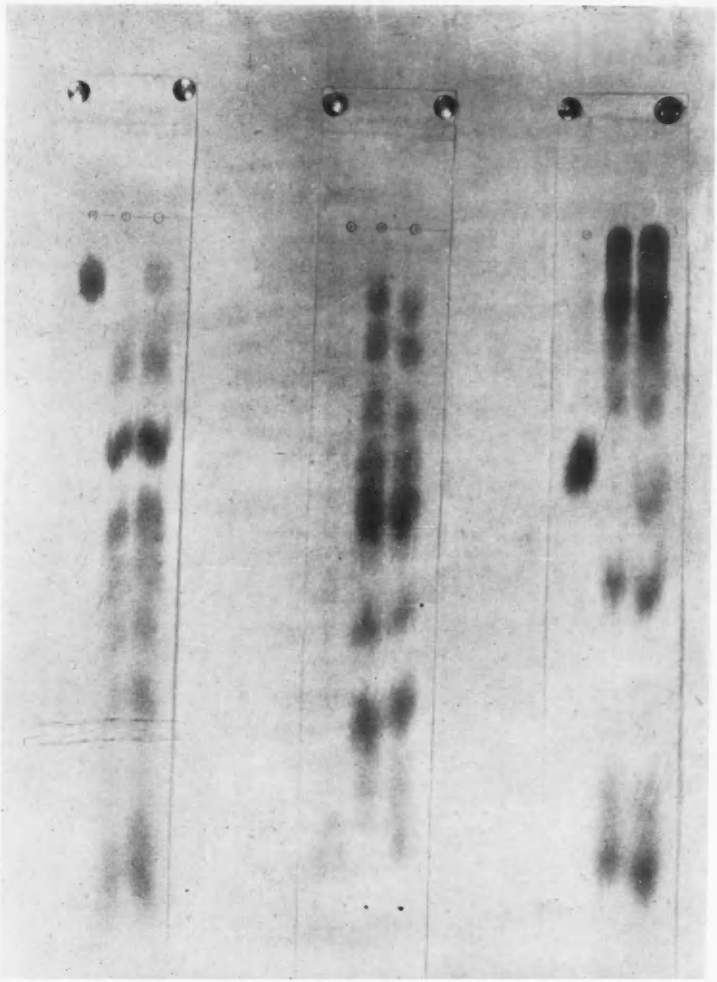

a *b* *c*

PLATE 5. One-dimensional chromatograms on Whatman no. 4 paper. In each set of three, the solutions were applied in the circles along the line near the top of the strip. The amino-acid to be tested (5–10 μg.) was applied at the circle on the left, a hydrolysate of virgin root wool (100–150 μg.) in the middle circle, and a hydrolysate (100–150 μg.) containing the added amino-acid in the right-hand circle. After development of the chromatograms with the solvent, saturated with water, the strips were dried and treated with ninhydrin.

a. Cysteic acid; wool hydrolysate; hydrolysate of commercial dry chlorinated wool. Chromatograms developed in phenol-coal gas-NH_3 for 17 hr.

b. Thiazolidine carboxylic acid; wool hydrolysate; wool hydrolysate plus thiazolidine carboxylic acid. Chromatograms developed in S-collidine for 17 hr.

c. *S*-methyl cysteine; wool hydrolysate; hydrolysate of reduced, methylated wool. Chromatograms developed in *n*-butanol-HCN for 24 hr.

R. CONSDEN, A. H. GORDON AND A. J. P. MARTIN—THE IDENTIFICATION OF AMINO-ACIDS DERIVED FROM CYSTINE IN CHEMICALLY MODIFIED WOOL

PLATE 6. Two-dimensional chromatogram of a hydrolysate of wool tips (360 μg. protein) on Whatman no. 4 sheet. Hydrolysate applied at circle. Run with collidine for 39 hr., then in a direction at right angles with phenol for 17 hr. in an atmosphere of coal gas and NH_3. The key diagram (Text-fig. 1) shows the positions of the amino-acids in virgin root wool (open circles). The positions of cysteic acid, djenkolic acid, lanthionine and thiazolidine carboxylic acid are indicated by dark circles. (*S*-methyl cysteine would be superimposed on valine.)

The Influence of Hydrolysates of Hyaluronate upon Hyaluronidase Production by Micro-organisms

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(*Received* 25 *April* 1946)

It has been shown (McClean & Hale, 1941; McClean, 1941; Rogers, 1945) that hyaluronidase production by certain streptococci and *Cl. welchii* is increased adaptively by the inclusion of hyaluronate in growth media for these organisms. Enzyme production by streptococci is similarly increased by including the products formed from hyaluronate by the action of testicular hyaluronidase at pH 7·0, but not by the addition of substances resulting from the hydrolysis of the polysaccharide by hyaluronidase derived from streptococci (Rogers, 1945).

The present paper extends these observations to a study of the influence of other hydrolysates of hyaluronate upon hyaluronidase production by *Strep. haemolyticus* Group C (Lancefield) type 7 (Griffiths), in the sequel called the streptococcus C7, and by a strain of *Cl. welchii*. The relation between the amount by which enzymic hydrolysates stimulate hyaluronidase formation and the degree to which they inhibit the viscosity reducing activity of the enzymes has also been studied. A direct relation between these two values might be expected if Yudkin's (1938) mass action theory for the mechanism of adaptive enzyme formation is applicable to the production of hyaluronidase by micro-organisms.

METHODS

The medium and technique for growing the organisms used, together with the methods of estimating growth and the amount of enzyme produced, were as previously described (Rogers, 1945). Reducing sugars were estimated by Somogyi's (1937) reagent standardized with an equimolar mixture of *N*-acetylglucosamine and glucurone. Glucosamine was estimated by Elson & Morgan's (1933) method, *N*-acetylglucosamine by Morgan & Elson's (1934) method, and nitrogen by the micro-Kjeldahl method.

Preparation of the enzymes used for hydrolysis

(1) *Testicular hyaluronidase*

(*a*) Crude preparation. Fresh bulls' testes from the abattoir were ground with five times their weight of distilled water. The pulp obtained was filtered through glass-wool and the filtrate frozen at −10°. Measured samples of this extract were thawed and spun at 2–3000 r.p.m. for 15 min. on an angle centrifuge immediately before use. The enzyme titre of the supernatants was 290 viscosity reducing units (v.r.u.; McClean & Hale, 1941; McClean, 1943).

(*b*) Partially purified preparations were made by the method of Madinaveitia (1941) or by that of Morgan & McClean (1932).

(2) *Streptococcal hyaluronidase*

(*a*) Crude preparation. A filtrate from a culture of the streptococcus C7 grown in a peptone medium was saturated to 70% with $(NH_4)_2SO_4$. The precipitate obtained was collected and dialyzed in the presence of toluol against running tap water until free of $(NH_4)_2SO_4$. This solution was used as the crude hyaluronidase preparation.

(*b*) Partially purified preparation. The streptococcus C7 was grown for 24 hr. in the casein hydrolysate medium B (Rogers, 1945) containing 0·2 g./100 ml. crude potassium hyaluronate. The organisms were removed by mixing the culture with kieselguhr and filtering through fluted papers. The filtrate was then dialyzed against running tap water for 24 hr. in the presence of toluol. The dialysate was adjusted to pH 5·6 with 2N-acetic acid and 50 ml./l. of a solution of dialyzed iron (British Drug Houses Ltd.) added. The precipitate of $Fe(OH)_3$ was allowed to remain in contact with the dialysate for 15–20 min. after which it was removed by centrifugation through a Sharples supercentrifuge. The enzyme was eluted from the $Fe(OH)_3$ with 0·2M-Na_2CO_3. It was sometimes necessary to perform as many as five elutions in order to recover the maximum amount of enzyme. The yield from this process varied from 20 to 50%; further attempts are being made to improve the method.

(3) Cl. welchii *hyaluronidase*

(*a*) Crude preparation. This was made from the filtrate from a culture of *Cl. welchii* type A, strain 107, by the same technique as that used for the crude preparation of hyaluronidase from the streptococcus C7.

(*b*) Partially purified preparation. A concentrated filtrate from cultures of *Cl. welchii*, strains 107, which had already been fractionated by $(NH_4)_2SO_4$ precipitation was kindly provided by Dr W. E. van Heyningen (The Wellcome Physiological Research Laboratories). This concentrate had an enzyme titre of 122,000 v.r.u. and contained 37·8 mg. N/ml.; 10 ml. were mixed with buffer (12 ml. of McIlvaine buffer at pH 4·6) and water (20 ml.) and passed through a column 8 cm. long × 2 cm. diameter of Al_2O_3 (Savory and Moore Ltd. London), using very gentle suction. The column was washed with 50 ml. of water, and phosphate buffer (0·1 M at pH 7·0 or 0·1 M-Na_2HPO_4) then passed down the column. Successive fractions of 1–3 ml. of the effluent were taken and tested by the mucin clot prevention test (McClean, 1943); the most active fractions were checked by the more exact v.r.u. method. The passage of buffer was continued until no further enzyme could be detected in the effluent. Using this procedure it was found that about 50%

of the enzyme applied to the column was obtained in three of the fractions with a total volume of 3–4 ml. These fractions contained a total of only 4% of the original N in the preparation. Similarly promising results were obtained with four other crude preparations of *Cl. welchii* hyaluronidase. One preparation, however, failed to give a sharp band, and the method in its present form is not applicable to the purification of streptococcal hyaluronidase. Further studies of the chromatographic method for the purification of this group of enzymes are now to be undertaken.

EXPERIMENTAL

The hydrolysis of hyaluronate by hyaluronidase of different degrees of purity

The streptococcal enzyme used in the previous work (Rogers, 1945) was a relatively crude preparation, whereas the testicular hyaluronidase used here had been partially purified by Morgan & McClean's (1932) method. It was previously suggested (Rogers, 1945) that the different behaviour of the hydrolysates produced by these two enzyme preparations might be explained by this difference in degree of purification. To test this hypothesis, the following series of hydrolysates was made:

Pairs of enzyme preparations of differing purity (as indicated by the ratio of enzyme activity to their content of total N) were used to hydrolyze solutions of purified potassium hyaluronate under the following conditions:

The hyaluronate solution (10 ml. of 0·5 g./100 ml. solution) was mixed with 2·5 ml. of McIlvaine buffer (0·1 M, pH 7·0 and containing 0·6 M-NaCl) and incubated at 37° with the hyaluronidase preparations indicated in Table 1, until no further reducing sugar was liberated; chloroform was present throughout to prevent bacterial growth. From 600 to 1200 v.r.u. of hyaluronidase were used for the 12·5 ml. of the substrate buffer mixture.

The hydrolysates were sterilized and the enzymes they contained inactivated by heating in a boiling water-bath for 15 min.; the pH of the solutions was first lowered to 6·5. This process produced very much less caramelization than did autoclaving (Rogers, 1945) and the enzymes were always completely inactivated. Sterility was tested by adding 1 ml. of the treated hydrolysates to 4 ml. of a glucose-peptone broth and incubating this mixture for 48 hr. at 37°. An occasional hydrolysate contained resistant organisms and was autoclaved for 15 min. at 115°. The technique for testing the influence of the hydrolysates upon hyaluronidase production by the streptococcus C 7 was as previously described (Rogers, 1945). Tubes of medium were also supplemented by buffer and inactivated enzyme as controls.

All the hydrolysates listed in Table 1 were not tested in the same experiment. For this reason both the absolute stimulation (Rogers, 1945), which varies somewhat with different seed cultures, and the ratio of this to the stimulation caused by an equivalent concentration of unhydrolyzed hyaluronate, are given. This ratio has been found to be constant for any one hydrolysate from experiment to experiment. Table 1 shows the influence upon hyaluronidase production by the streptococcus C 7 of adding the various enzymic hydrolysates of hyaluronate to the casein hydrolysate medium B (Rogers, 1945).

Irrespective of the purity of the enzyme preparations used, the hydrolysis products formed by testicular hyaluronidase cause the same degree of stimulation, within the experimental error of the method, as an equivalent concentration of unhydrolyzed hyaluronate. The streptococcal and *Cl. welchii* type A strain 107 hyaluronidase preparations all left hydrolysis products which had little influence upon hyaluronidase production by the streptococcus C 7. Hence the different behaviour of the hydrolysates produced using testicular hyaluronidase and these two bacterial enzymes cannot be explained on the basis of the purity of the enzyme preparations.

Table 1. *The influence of various enzymic hydrolysates of hyaluronate upon hyaluronidase production by the streptococcus C 7*

Source of enzyme used for hydrolysis	Preparation	Purity of enzyme (v.r.u./mg. N)	Hydrolysis* of substrate (%)	Stimulation (S)	S/W†
Bull testes	A‡	—	57	3·12	0·80
	B§	120–320	54·2–78·0	2·1–4·7	1·03–1·09
Cl. welchii	A‡	3470	65	0	0
	B§	58,100	54·2	0·11	0·04
Streptococcus C 7	A‡	520	83	0·30	0·26
	B§	4550	45·8	0·41	0·17

* Measured by the release of reducing sugars from the polysaccharide.

† $S/W = \dfrac{\text{Stimulation by hydrolysate}}{\text{Stimulation by an equivalent amount of unhydrolyzed hyaluronate}}$.

‡ Crude preparation. § Partially purified preparation.

Hydrolysis of hyaluronate by mild treatment with acids

Hydrolysis of hyaluronate by strong acids such as 2–4N-HCl yields glucosamine, acetic acid and various degradation products of glucuronic acid. Thus such treatment not only hydrolyzes the glycosidic bonds of the polysaccharides, but disrupts the constituent sugar derivatives. The mixtures obtained were not suitable for an investigation of the influence of acid hydrolysates of hyaluronate upon hyaluronidase production. Hydrolysis of hyaluronate at the temperature of a boiling water-bath by N-acetic acid yields *N*-acetylglucosamine as determined by the Morgan & Elson (1934) test (Rogers, 1946). After 30 hr. treatment, 100% of the *N*-acetylglucosamine predicted to be present in the polysaccharide from its hexosamine content had been liberated. Some destruction of glucuronic acid does take place during this treatment. Experiments, however, using more dilute acid, did not result in less destruction of glucuronic acid for an equivalent release of *N*-acetylglucosamine.

The addition of N-acetate solution to the test medium somewhat suppressed hyaluronidase production by the streptococcus C 7 and acetic acid was thus an undesirable hydrolytic agent. An oxalic acid-sodium oxalate mixture at pH 2·3 was found to be satisfactory and the oxalate could be removed as the calcium salt before the hydrolysates were added to the test medium. The conditions of hydrolysis were as follows:

Purified hyaluronate (0·2 g.) was dissolved in 40 ml. of distilled water. Oxalic acid (2·522 g.) was then added, dissolved, and the pH of the mixture adjusted to 2·5 with N-NaOH, using a glass electrode. A precipitate of sodium oxalate formed and was removed by filtration; the volume of the filtrate was adjusted to 50 ml. with washings from the precipitate. The solution was then heated under reflux in a boiling water-bath and samples (12 ml.) were withdrawn at appropriate time intervals. These were cooled and approximately 0·5 g. of $CaCO_3$ added in small portions with mechanical stirring. The calcium oxalate and unchanged carbonate were then removed by centrifuging and the small amounts of oxalate remaining in the supernatant fluid were precipitated by the addition of $CaCl_2$ solution. Glucosamine was estimated before and after removal of the oxalate to check that no adsorption of the hydrolytic products by the precipitated Ca salts had taken place; *N*-acetylglucosamine was determined in the final supernatant liquids. The hydrolysates were neutralized to pH 7·0 and sterilized by autoclaving at 115° for 15 min. during which no caramelization took place.

The influence of the hydrolysates upon hyaluronidase production by streptococcus C 7 was tested in the usual manner. Table 2 shows that the ability of hyaluronate to stimulate hyaluronidase production by the streptococcus C 7 was progressively destroyed during hydrolysis. It will be seen from Table 2 that when 70% of the calculated amount of *N*-acetylglucosamine had been released, the hydrolysis products had no influence on enzyme formation. This hydrolysis by acids resembles that by streptococcal and *Cl. welchii* hyaluronidase. It may be noted that the degree of stimulation caused by the acid hydrolysates is consistently less than that to be expected from the amount of *N*-acetylglucosamine calculated to be still in unhydrolyzed units (i.e. not reacting to give colour with the *p*-dimethylaminobenzaldehyde reagent under the conditions used by Morgan & Elson, 1934).

Table 2. *The influence of hydrolysis of hyaluronate by an oxalic acid-sodium oxalate mixture at pH 2·5 upon the ability of the hydrolysis products to influence hyaluronidase formation by the streptococcus C 7*

Hydrolysis* (%)	Stimulation (Observed)	Stimulation (Calculated)†
0	4·30	—
16·7	2·90	3·50
43·7	1·46	2·60
70	0	1·29

* Measured by the release of *N*-acetylglucosamine.
† Calculated from the amount of bound *N*-acetylglucosamine still present in the hydrolysate.

The influence of enzymically hydrolyzed hyaluronate on hyaluronidase production by Cl. welchii *type A strain A 118d*

Gale & van Heyningen (1942) found that maximum amounts of hyaluronidase were produced by a strain of *Cl. welchii* type A after 6–7 hr. growth in a peptone medium. They stated, however, that more hyaluronidase was sometimes found in 24 hr. cultures. Hahn (1945) working with a peptone medium, and Byers, Tytell & Logan (1945) using a meat digest medium containing hyaluronate, both found that the amounts of enzyme in filtrates from *Cl. welchii* cultures were greater when the incubation period was increased from 24 hr. to 5 days.

In the present work it was found, with strain A 118*d* of *Cl. welchii* and the casein hydrolysate Medium A (Rogers, 1945), that considerable variation occurred in the rate of production of hyaluronidase. Whereas in some experiments maximum amounts of this enzyme were produced after only 18 hr. incubation, in others hyaluronidase formation continued up to 4–5 days' incubation. This slow production of the enzyme was very much more pronounced in media supplemented by hyaluronate. Enzyme formation by the organisms growing in the unsupplemented media was usually maximal after 18–40 hr. incubation. These observations made necessary considerable changes in the technique

hitherto found satisfactory for examining the influence of the composition of the growth medium upon hyaluronidase production by organisms. The new technique was as follows:

Hyaluronidase production in all the cultures in an experiment was measured at 24 hr. intervals until no further increase in enzyme formation could be detected. The mucin clot prevention test (McClean, 1943) was used in the preliminary work. The cultures at the end of this period were centrifuged and the amount of hyaluronidase was estimated in the supernatants by the more accurate V.R.U. method. When hyaluronidase production had ceased, the bacterial cells had very largely autolyzed. It was for this reason not possible to estimate the mass of bacterial growth which had occurred. Consequently the stimulation caused by both hyaluronate and the preparations of enzymically hydrolyzed hyaluronate was calculated simply from the amount of enzyme in the culture supernatants without correction for variations in growth.

The influence of the concentration of hyaluronate upon hyaluronidase production by Cl. welchii *strain A 118d*

The stimulation of hyaluronidase production by the streptococcus C 7 was directly proportional to the concentration of hyaluronate present in the medium, up to 0·6 g./100 ml. (Rogers, 1945). The influence of the polysaccharide content of the medium upon hyaluronidase production by *Cl. welchii* strain A 118*d* was investigated as follows:

Volumes of a sterile solution of crude potassium hyaluronate (0·5 g./100 ml.) were added to 5 ml. of double-strength test medium A (Rogers, 1945) to give final concentrations of 0·03, 0·06, 0·09, 0·125, and 0·180 g./100 ml. of the polysaccharide. Sufficient sterile distilled water was then added to adjust the volumes in the tubes to 10 ml. The media all contained glucose (0·25 g./100 ml.) and each tube was inoculated with one drop of an 18 hr. meat culture of the organism. Two tubes of each test medium were inoculated.

This experiment was repeated several times. There was considerable variation in the degree of stimulation caused by the same amount of hyaluronate from experiment to experiment. Fig. 1 shows the final amounts of enzyme in the cultures from two such experiments; the variation was probably due to differences in the seed culture rather than to uncontrolled variation of the medium since the same batch of medium and of hyaluronate solution was used for two experiments quoted and results for the duplicate pairs of cultures in different experiments agreed within 10%.

In the next series of experiments enzymically hydrolyzed hyaluronate was added to the culture medium. The technique for the preparation of the hydrolysates was as already described when investigating their action upon the streptococcus C 7. Partially purified enzyme preparations were used for hydrolysis. Each 10 ml. of casein hydrolysate growth medium contained 2·0 ml. of the hydrolysates. The technique for examining hyaluronidase production by *Cl. welchii* strain A 118*d* was as already described.

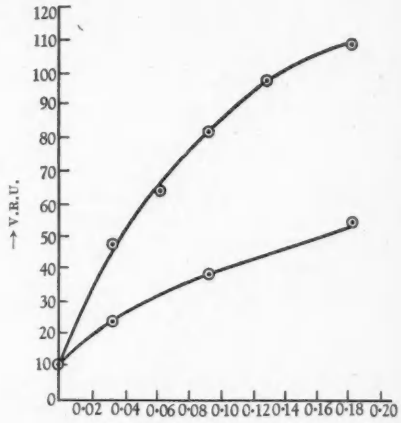

Fig. 1. The influence of the concentration of hyaluronate (g./100 ml.) present in the casein hydrolysate medium on hyaluronidase production measured in V.R.U. (McClean & Hale, 1941) by *Cl. welchii* type A strain A 118*d*. The results of two separate experiments are plotted in the figure; different seed cultures were used for these two experiments.

The products formed by testicular enzyme stimulated hyaluronidase production. Table 3 shows that hydrolysates prepared using purified streptococcal and *Cl. welchii* enzymes also increased enzyme production. The stimulation in the medium containing hyaluronate hydrolyzed with streptococcal hyaluronidase is sufficiently high to be significant. The result in the medium supplemented

Table 3. *The influence of various enzymic hydrolysates of hyaluronate upon hyaluronidase production of* Cl. welchii *strain A 118d*

Source of enzyme used for hydrolysis	Hydrolysis* of substrate (%)	Stimulation	*S*/*W*†
Bull testes	30·5	3·49	1·16
Cl. welchii	54·2	1·35	0·44
Streptococcus C 7	45·8	2·44	0·81
0·06 g./100 ml. unhydrolyzed hyaluronate		1·94	—
0·12 g./100 ml. unhydrolyzed hyaluronate		3·62	—

* Measured by the release of reducing sugars from the polysaccharide.

† Stimulation observed/stimulation by an equivalent concentration of unhydrolyzed hyaluronate.

with the products formed by the *Cl. welchii* enzyme is less definite but is probably significant. Owing to the less accurate nature of the test that had to be used for this organism, however, it is not possible to be quite certain of the significance of this latter result.

The inhibition of streptococcal and Cl. welchii *hyaluronidase*

Yudkin (1938) suggested that the adaptive formation of enzymes by micro-organisms was a mass-action phenomenon. In this theory the small amounts of enzyme initially formed by the organism combine with the substrate leading to the production of more enzyme. As emphasized by Stephenson (1939) substances which competitively inhibit an enzyme should also stimulate its microbiological production if Yudkin's hypothesis is correct. McClean (1942) showed that heparin and chondroitin sulphuric acid both inhibited the action of purified testicular hyaluronidase upon hyaluronic acid and upon the capsules of Lancefield group A streptococci. These capsules are known to consist of hyaluronate.

It has been confirmed by viscosimetric test that both heparin and chondroitin sulphuric ester inhibit the action of a partially purified preparation of streptococcal hyaluronidase upon hyaluronic acid, e.g. 0·21 mg./ml. of heparin (Roche Products Ltd.) in the final solution used for the viscosity reduction test (McClean & Hale, 1941; McClean, 1943) reduced the titre of the enzyme from 256 to 126 V.R.U. It was, however, previously reported (Rogers, 1945) that neither of these polysaccharides increases hyaluronidase production by the streptococcus C 7.

The influence of the heated enzymic hydrolysates of hyaluronate upon the action of streptococcal and *Cl. welchii* hyaluronidase has now been examined as follows:

Mixtures containing increasing ratios of amounts of enzyme to hydrolysate were prepared and then diluted so that 0·5 ml. of the mixture would reduce the viscosity of the batch of hyaluronate used for viscosimetric determinations to half its initial value in approximately 20 min. under the usual conditions of the test. The apparent strength of the enzyme in the mixture was calculated in the usual manner (McClean & Hale, 1941).

Fig. 2 shows the results obtained when two partially purified enzyme preparations were used in these tests. The actual degree of inhibition varied according to the nature of the enzyme preparation. For example, there was very little inhibition by either of the hydrolysates when an unmodified filtrate from a streptococcus C 7 culture was used as a source of enzyme. The somewhat greater inhibition of streptococcal hyaluronidase caused by the hydrolytic products formed by testicular enzyme appeared significant. When, however, the degree of inhibition of a streptococcal enzyme preparation by the dialysate and diffusate from this hydrolysate was tested it was found that 85 % of the inhibiting substance was present in the diffusate. It was previously found (Rogers, 1945) that all the substance stimulating hyaluronidase production by the

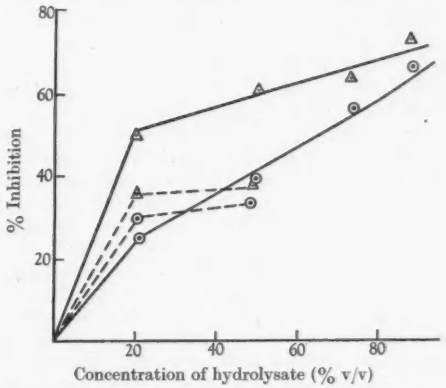

Fig. 2. The influence of increasing concentration (% v/v in the mixture diluted for test) of hyaluronate hydrolyzed by different enzyme preparations on the activity of hyaluronidase produced by the streptococcus C7 and by *Cl. welchii* strain S107. (1) Hydrolysis by testicular hyaluronidase: effect upon (*a*) streptococcal enzyme △—△, (*b*) *Cl. welchii* enzyme △---△. (2) Hydrolysis by streptococcal hyaluronidase: effect upon (*a*) streptococcal enzyme ⊙—⊙, (*b*) *Cl. welchii* enzyme ⊙---⊙.

streptococcus C 7 was present in the dialysate, the diffusate having no influence upon enzyme formation. Attempts were made to determine whether the inhibition of enzyme activity caused by the hydrolysate and diffusate were competitive. The viscosity reduction test, however, does not lend itself easily to such experiments since increased substrate concentration leads to very long times of flow of the substrate-enzyme mixture through the viscometer and great inaccuracies in determining enzyme titres. The results, however, did indicate a somewhat decreased degree of inhibition when the substrate concentration was raised from 0·12 to 0·5 g./100 ml. From these experiments it does not seem that Yudkin's (1938) hypothesis can be applied to the microbiological production of hyaluronidase.

DISCUSSION

The influence of enzymic hydrolysates of hyaluronate upon the production of hyaluronidase by those organisms which form this enzyme adaptively appears to be a delicate qualitative method for

distinguishing between the hydrolysates. The substances obtained when the polysaccharide is broken down by testicular hyaluronidase at pH 7·0 are still as active in stimulating enzyme production by both multiplying streptococci and by *Cl. welchii*, as is the undegraded hyaluronate. Chemically these hydrolysates are distinguished (Rogers, 1946) from those obtained by using streptococcal or *Cl. welchii* hyaluronidase, by the presence of oligosaccharides not diffusible through cellophan, but which show very considerable reducing power towards the alkaline copper reagent of Somogyi (1937). The results suggest that the smaller but still relatively complex molecules of oligosaccharide, such as are present in these hydrolysates, are as effective as the molecules of undegraded polysaccharide in stimulating the organisms. If the hyaluronate molecules are attacked by the enzymes in the testicular preparations primarily at their ends, then the oligosaccharides remaining would be in the same molecular concentration as the original polysaccharide. This might explain the quantitatively equivalent response of the organisms to the hydrolyzed and unhydrolyzed hyaluronate. It has also been shown (Rogers, 1946) that after hydrolysis of hyaluronate at pH 7·0 by streptococcal hyaluronidase, complex but smaller, diffusible molecules remain. These hydrolysates have little or no influence upon hyaluronidase production by streptococci. Thus it would seem that relatively large molecules are necessary to stimulate these organisms. The smaller molecules are, however, probably effective for *Cl. welchii*. This result would appear to indicate an interesting difference in the mechanism of adaptive enzyme formation by the two organisms.

The comparative rates of production of hyaluronidase by *Cl. welchii* and streptococci are particularly interesting in light of the statement by Meyer, Hobby, Chaffee & Dawson (1940) that no hyaluronidase can be extracted from the cells of *Cl. welchii* grown on a medium not containing hyaluronate, but that, as confirmed by general experience, the culture filtrates are a potent source of enzyme. They found that the reverse situation was true for pneumococcal cultures. It appears from the results in the present paper, however, that in some cultures most of the enzyme production by *Cl. welchii* takes place while the organisms are autolyzing. Casein hydrolysate media are the only ones that have been used but the results of Hahn (1945) and Byers *et al.* (1945) indicate that enzyme must also appear in culture-filtrates during autolysis of organisms grown on a peptone medium; but again Gale & van Heyningen's (1942) results suggest that this may only occasionally be true. It would be of interest to know more of the conditions which determine whether the enzyme shall be secreted freely during the growth phase of the organisms or only released on autolysis of the cells. With the strain of streptococcus used here (C7), and grown on the casein hydrolysate growth medium, hyaluronidase was always produced during the active growth of the organisms, although Meyer, Chaffee, Hobby & Dawson (1941) found that hyaluronidase was present in both the cells and the cell-free supernatant fluid from cultures of streptococci.

SUMMARY

1. Potassium hyaluronate, hydrolyzed by crude or purified testicular hyaluronidase, can stimulate hyaluronidase formation by the streptococcus C7 or *Cl. welchii* strain A118*d* equally as well as the unhydrolyzed polysaccharide.

2. Potassium hyaluronate hydrolyzed by streptococcal or *Cl. welchii* enzymes does not stimulate hyaluronidase production by the streptococcus C7. The streptococcal hydrolysate does, however, stimulate hyaluronidase production by *Cl. welchii* A118*d*, whilst the *Cl. welchii* hydrolysate has a small but probably significant influence on this organism.

3. Hydrolysis of hyaluronate by mild treatment with acids destroys the ability of the polysaccharide to enhance hyaluronidase production by the streptococcus C7.

4. The ability of substances to inhibit competitively the activity of hyaluronidase is not correlated with their ability to stimulate microbiological formation of the enzyme.

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On Phytic Acid, its Importance in Metabolism and its Enzymic Cleavage in Bread Supplemented with Calcium

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(*Received* 20 *February* 1946)

I. IMPORTANT PROPERTIES OF PHYTIC ACID

Phytic acid, a hexaphosphoric ester of inositol, is found in all plant seeds examined, in grains as well as in oilseeds, and undoubtedly represents a store of phosphoric acid that is hydrolyzed in sprouting. For this reason green plants never contain considerable amounts of phytic acid. The cleavage in the seed is evidently caused by a specific enzyme called phytase, but other phosphatases from plant tissue are able to split the substance to some degree.

In most cases no phytase activity is found in resting seeds. The enzyme seems to be formed during sprouting of the seeds. Wheat and rye, and to a certain extent barley and buckwheat, are, however, exceptions from this rule. If the whole flour of these grains is suspended in a buffer solution at pH 5 and left at a temperature of 40° for 2 hr. all the phytic acid in wheat and rye, and a considerable part of the acid in barley and buckwheat, is split into inositol and free phosphoric acid. In flours of any other seeds examined no cleavage of phytic acid takes place under these conditions. Experiments with pure phytase preparations have proved the conditions mentioned to be optimal, and confirm the conclusion that these grains do not contain phytase. Table 1 shows the results of investigations by Pedersen (1940) in Møllgaard's laboratory.

Total P was in the experiments of Pedersen and in other experiments referred to in this paper determined by destruction of the substance with concentrated sulphuric and nitric acids and precipitation as ammonium phosphomolybdate; this was heated at 550° to form phosphomolybdic acid (Hansen & Græsholm, 1935).

The phytic P was determined by extraction with 0·5N-HCl and precipitation as iron phytate; this was decomposed with NaOH, and the P in the dissolved sodium phytate was determined according to the method for total P (Pedersen, 1940).

Ca was determined by destruction with concentrated sulphuric acid, dissolution of the ash in diluted hydrochloric acid and precipitation as oxalate, which was titrated with permanganate (Hansen & Græsholm, 1935).

Table 1. *The phytic acid and phytase content of certain cereals and seeds*

	Total P (%)	Phytic P (%)	Phytic P (as % of total)	Phytic P split in 2 hr. by the naturally present phytase (%)
Wheat	0·319	0·273	86	100
Wheat bran	1·099	0·944	86	100
	1·120	0·965	86	100
Rye	0·342	0·250	73	100
Barley	0·281	0·182	65	69
	0·322	0·205	64	94
Buckwheat	0·304	0·192	63	55
Maize	0·255	0·205	80	0
	0·330	0·270	82	2
	0·248	0·199	80	4
Oats	0·345	0·228	66	8
Cotton-seed meal:				
Brazil	1·047	0·812	78	4
Siam	1·446	1·183	82	3
Linseed meal	0·989	0·772	78	2
Ground-nut meal	0·992	0·529	76	0
Coconut meal	0·505	0·258	51	0
Palm-kernel meal	0·576	0·481	84	0
Rape-seed meal	0·814	0·649	80	10
Sunflower-seed meal	0·944	0·803	85	0
Soya-bean meal	0·629	0·426	68	0

Table 1 shows that a very considerable part of the total P in seeds is present as phytic acid; in the grains used for human food this amounts to 75–85%. Maize and oats contain no phytase, but Pedersen (1940) has shown that a considerable part of their phytic acid can be split if the flours are suspended in a solution of phytase extracted from wheat bran, and kept under optimal conditions.

Phytic acid forms an interesting calcium salt. In Fig. 1 is shown a titration curve for calcium phytate. It was made by dissolving equivalent amounts of sodium phytate and calcium chloride in water at pH 2 and titrating with 0·1N-NaOH, the potentials being measured with the glass electrode. The molarity of the solution was 0·0067 at the beginning of the titration. The figure shows that the curve has two turning points. At pH 3·7 a precipitate of calcium phytate was formed, probably a basic salt. The analysis of the substance showed a P : Ca ratio of 1·25 : 1.

In his investigation of the solubility of calcium phytate Hoff-Jørgensen (1944) found that a salt of a constant composition was precipitated within the pH interval of 4·6–6·9, the P : Ca ratio being approximately 1·2 : 1. This seems to be in fair agreement with our result and corresponds to the empirical formula: $C_6H_2O_{24}P_6Ca_5$, which means, that the salt formed is pentacalcium phytate.

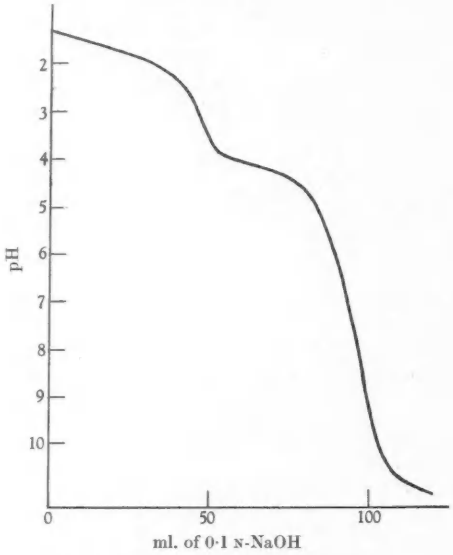

Fig. 1. Titration curve of calcium phytate.

It is important, that this salt is precipitated at a rather acid reaction, because it means that the presence of phytic acid in the intestines may seriously interfere with the absorption of calcium, even in the first parts of the small intestine, where the reaction corresponds to a pH of 4–5.

On the other hand we have found that certain organic oxy-acids forming complex calcium salts are able to shift the precipitation point of calcium phytate to a more alkaline reaction. Fig. 2 shows the effect of increasing concentrations of lactic, tartaric, citric and gluconic acids. It will be seen that tartaric and citric acids especially, in higher concentrations, effect a very large displacement of the pH of precipitation. Lactic acid also has a considerable influence. This is particularly remarkable, since considerable amounts of lactic acid are produced by fermentation in the intestines of many animals, and to some extent in those of man. These results have been confirmed by Christensen (1944), who in the laboratory of Møllgaard measured quantitatively the effect of oxy-acids on the solubility of pentacalcium phytate. An excess of this substance was shaken for 24 hr. at a temperature of 20° with solutions of sodium chloride, tartaric acid and citric acid. From the suspension 10 ml. were filtered and the contents of Ca and P

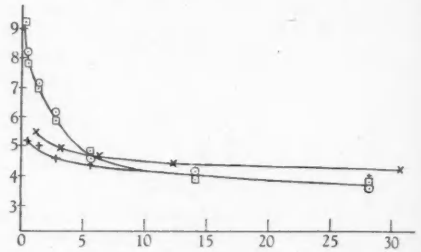

Fig. 2. The influence of oxy-acids upon the precipitation point of calcium phytate. The abscissa represents the quotient (m-equiv. phytate : m-equiv. oxy-acids) × 10, the ordinate the pH at which precipitation of calcium phytate takes place. × lactic acid, ⊙ tartaric acid, ⊡ citric acid, + gluconic acid.

determined. The results are given in Fig. 3. It is evident that the two acids increase enormously the solubility of the phytate. The same effect was found with $Ca(IO_3)_2.6H_2O$, so that it presumably takes place with any insoluble calcium salt, the

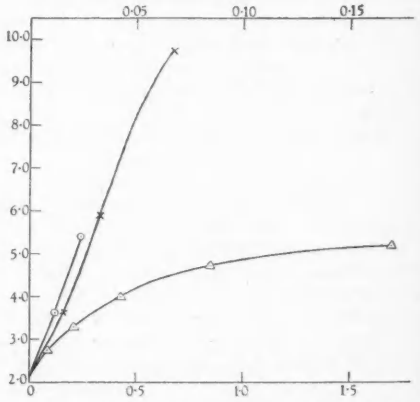

Fig. 3. The influence of oxy-acids on the solubility of calcium phytate. The abscissa represents the molar concentrations of sodium chloride and of tartaric and citric acid, the ordinate gives the concentration of calcium phytate in g./l. △ NaCl, × tartaric acid, ⊙ citric acid.

insoluble phosphates included. The influence of oxy-acids on calcium absorption may therefore be very far-reaching. The old question of the importance of fatty acids for this absorption may find its solution in that effect.

Phytic acid also forms insoluble iron salts. The exact composition of these salts is not known at present, but McCance, Edgecombe & Widdowson (1943) have demonstrated that the ferrous as well as the ferric ion is precipitated by sodium phytate at a pH of 6·5 and that the precipitate always contains more phosphorus than the equivalent of iron, being presumably a double phytate of sodium and iron. This means that both ferrous and ferric phytates are more insoluble at the pH obtaining in the largest part of the small intestine, than the corresponding phosphates or hydroxides. Phytic acid may therefore also interfere with the absorption of iron from the intestine.

II. THE REACTION IN THE INTESTINE

As far as we know an element must be in solution in order to be absorbed from the intestine. As the solubility of calcium and phosphate ions is strictly dependent on the reaction of the solvent, the pH in the contents of the different parts of the intestinal canal must be of decisive importance in the absorption of these elements when they are present together. The same holds presumably for iron, because it forms insoluble precipitates with phosphoric acid in neutral solution. It is therefore regrettable that the whole question of the reaction in the intestinal canal of animals and man has not yet been given the attention it deserves. It seems that up to now the only extensive investigation was that done by Hagens in the laboratory of Møllgaard. Hagens measured the reaction in the different parts of the intestine of pigs under varying conditions of feeding. Because of his unfortunate death during the German occupation, his work cannot yet be published in full. Only part of it has appeared (Hagens, 1943). In Tables 2–5 are given the results of his unpublished measurements on pigs under normal conditions of feeding. All the values were measured with the glass electrode on samples taken from tubes inserted in the stomach, the duodenum, the jejunum and the ileum. Full details of this technique will soon be published by Borch-Madsen (1946). In the jejunum the tubes were set 3–4 m., in the ileum 13–15 m., from the pylorus. The measuring took place immediately after the sample had been taken. In some of the animals the results were controlled by direct measurement with glass electrodes in the tubes. In these cases a calomel electrode was connected with the stomach and the rectum of the animal. The readings were about the same for both calomel electrodes and corresponded very closely with the results on the samples. In all cases the samples were taken at such time after feeding that the intestine in the section of the tube was known to be filled with food. Tables 2–5 give, therefore, values of pH during digestion.

Table 2. *pH in the stomach of the pig. Samples taken* 30 *min. after feeding*

	Samples from the outer liquid layers of food		Samples from the central mass of food	
Pig no.	No. of samples	pH range	No. of samples	pH range
T73	8	1·14–2·79		
T73	13	1·02–3·78		
T73	11	1·06–2·21	11	1·66–4·59
T73	16	1·35–3·48	16	1·79–5·29
T43	6	1·43–2·40	7	1·76–4·48
T43	5	1·81–3·66	4	3·61–4·19
T43	5	1·91–1·78	5	1·30–5·08
T43	5	1·34–3·03	5	1·34–3·99
T15	9	1·64–3·27		
T43	6	0·95–1·95	6	1·54–4·26

From Table 2 it appears that the pH in the central mass generally has a higher value than in the outer layers of the food. This was to be expected because the hydrochloric acid diffuses from outside into the food mass. In the liquid contents of the stomach the pH varies between 1 and 3.

Table 3. *pH in the duodenum of the pig. Samples taken* 45 *min. after feeding*

Pig no.	No. of samples	pH range
T73	11	2·46–6·15
T73	9	3·07–6·04
T73	11	3·30–6·10
T73	12	2·34–6·29
T43	6	4·03–5·04
T43	5	4·29–5·37
T43	6	3·97–5·97
T43	5	3·93–5·93
T15	14	4·04–5·66
T43	9	2·65–5·60
T33	6	3·91–6·12
T33	5	4·19–5·12
T33	7	4·31–5·36
T33	7	3·17–4·78
T6	8	3·60–5·60
T6	9	3·47–4·97

The values of pH in the duodenum vary widely. This would be expected because the stomach is emptied periodically. If the sample is taken immediately after the opening of the pylorus, the

Table 4. *pH in the jejunum of the pig. Samples taken* 3 *hr. after feeding*

Pig no.	No. of samples	pH range
T73	7	5·14–7·68
T73	5	5·30–5·61
T73	10	5·37–6·54
T73	8	5·66–6·03
T43	6	5·73–6·23
T43	5	5·92–6·38
T43	5	5·80–6·25
T43	6	5·98–6·52
T15	16	5·64–6·75
T43	7	5·59–6·35

pH will be considerably lower than later, when the contents of the duodenum have been partly neutralized by the pancreatic juice. The reaction, however, always remains acid, and corresponds generally to pH values between 3 and 6.

The variations of pH in the jejunum are much smaller than in the duodenum. It is further remarkable that the reaction still remains acid. On average the pH is between 5 and 6·5.

Table 5. *pH in the ileum of the pig. Samples taken 5 hr. after feeding*

Pig no.	No. of samples	pH range
T73	5	6·68–7·73
T73	2	7·25–7·51
T73	2	6·71–6·89
T43	6	6·72–7·39
T43	5	7·04–7·49
T43	4	7·35–7·57
T43	6	6·53–7·28
T15	6	6·75–7·33
T43	5	7·38–7·60
T33	4	7·20–7·36
T33	4	7·30–7·35
T33	4	7·10–7·46
T33	2	6·70–7·98

Table 5 shows that the reaction in the lower parts of the small intestine varies about the point of neutrality.

As a general result of these measurements, the following ranges for pH in the different parts of the intestine of pigs can be quoted:

Duodenum	3 –6
Middle of jejunum	5 –6·5
Lower part of ileum	6·5–7·5

This means that the reaction is *acid* in the largest part of the small intestine. Not until its end does the reaction become alkaline. Presumably the same holds for other mammals with a relatively short intestinal canal, man included.

III. ABSORPTION OF CALCIUM, PHOSPHATE AND IRON

The conditions of reaction mentioned are very important for the absorption of some of the most valuable foodstuffs. As considerable amounts of $HPO_4^=$ are not formed until the pH reaches the value of 6 and the quotient $\frac{H_2PO_4^-}{HPO_4^=}$ does not amount to 1 until the pH is 7, the calcium and the phosphate ions can remain in solution in the contents of a large part of the small intestine, and in these upper sections absorption of both ions can take place. In the lower parts of the small intestine these ions may be precipitated as $CaHPO_4$ and therefore withdrawn from absorption. Probably the same conditions are decisive for the absorption of the ferrous ion.

If, however, the intestine contains considerable amounts of phytic acid, Ca^{++} may be precipitated even at a pH of 3·7 and absorption very probably prevented within the whole range of the small intestine. To a certain extent the same may happen to the ferrous ion, which is precipitated as a double phytate at pH 6·5.

On the other hand, if the phytic acid is split to inositol and free phosphoric acid, the conditions are of course entirely altered. As far as we know the gastric and intestinal juices of the higher mammals including man contain no phosphatase that could effect the cleavage of phytic acid. The substance therefore cannot be digested unless the food itself contains a specific phytase. This may happen very frequently with domestic animals because some grains ordinarily used for feeding stuffs (wheat, rye, barley), and young green plants often contain considerable amounts of this enzyme. There is no doubt that an enzymic cleavage of phytic acid by means of food phytase takes place to a large extent in the stomach of pigs in the preliminary period of the digestion, where the pH amounts to 4·5. That this cleavage is of the greatest importance to the well-being of the animals will be shown later in this paper. In man, such cleavage of phytic acid by means of food phytase is not likely ever to take place, because he ordinarily eats food that in some way or another has been heated to a temperature that destroys any enzymic activity.

The precipitation of the calcium and ferrous ions, however, can be prevented if the intestinal food masses contain considerable amounts of oxy-acids, forming soluble complexes with these ions. In domestic animals this may be the case because they normally produce some lactic acid by fermentation in the stomach and the upper parts of the intestine, and lactic acid is known to shift the precipitation point of calcium phytate. If the lactic acid fermentation is sufficiently extensive it may reduce to a minimum the precipitation of phytates as well as phosphates of calcium and iron. Absorption of calcium ions would still take place because the equilibrium of calcium complex, calcium ion, phytate and phosphate ions is continuously readjusted as Ca^{++}, $CH_3CHOHCOO^-$ and $H_2PO_4^-$ are absorbed. The same holds for the ferrous ion. Under these conditions the harmful effect of phytic acid may be eliminated. Such conditions cannot be supposed to exist in man because the lactic acid fermentation in his intestinal canal is very slight so long as the secretion of hydrochloric acid in his stomach is normal. Unless he directly consumes oxy-acids, he cannot take advantage of their complex-forming capacity. In man, therefore, phytic acid may presumably unfold its whole harmful effect on the absorption of calcium and iron.

If no cleavage of phytic acid takes place, the phosphoric acid in it cannot be absorbed because, except for glucose- and glycerol-phosphoric esters, we know of no organic compound of phosphoric acid that is absorbed as such. In this case phytic acid presumably has another harmful effect, namely that its phosphoric acid is withdrawn from metabolism. If the food consists largely of grain products, a dangerous decrease of the phosphorus supply may result. The risk of this happening is definitely greater in the pig and in man than in most other mammals.

Further, there is very good reason to believe that the precipitation of calcium phytate in the small intestine may depress not only the absorption of calcium but also of phosphorus, by making the phytic acid inaccessible to the action of phytase, if this enzyme is present in the food. Under such conditions oxy-acids may promote absorption of phosphorus by bringing calcium phytate and, in the lower parts of the intestine, calcium phosphate into solution.

IV. EXPERIMENTAL EVIDENCE OF THE HARMFUL EFFECT OF PHYTIC ACID ON THE ABSORPTION OF CALCIUM AND IRON

A. *Review of literature.* Owing to the length of time during which British and Danish investigations on these problems have been out of touch the references in this paper to British work cannot be complete. If some important British work on this matter is not mentioned, it is earnestly hoped that the authors will understand that this omission was caused by wartime circumstances.

Harrison & Mellanby (1939) expressed the opinion that the pronounced rachitogenic effect of oatmeal was a consequence of the fact that phytic acid formed insoluble calcium salts, and that in this way the absorption of phosphoric acid as well as of calcium was seriously reduced. An experimental proof that phytic acid in the food really diminished the absorption of calcium was not published by these or any other authors writing on the subject at that time. Pedersen (1940), however, brought forward some evidence. In the course of extensive investigations of the rachitogenic effect of phytic acid, he found that the calcium content of the faeces of pigs fed a ration containing phytic acid was strictly correlated with the content of phosphoric acid. As the amount of undigested phosphoric acid in these experiments was shown to be approximately equivalent to the amount of phytic acid in the food, the amount of unabsorbed calcium was directly correlated with the content of phytic acid in the food. This finding afforded evidence that the amount of phytic acid in the food limits quantitatively the absorption of calcium by pigs.

In the same year Krieger & Steenbock (1940) published investigations on the availability of phytic acid phosphorus to rats. It is known that these animals produce some kind of 'phytase' in their intestinal canal, and therefore are able to split phytic acid to a certain extent. Using the percentage of bone ash as criterion, the two authors found that the availability of phytic phosphorus decreased markedly as the calcium content of the food increased, a fact that can hardly be explained otherwise than by assuming that calcium in the food prevents the splitting of phytic acid by precipitating it as an insoluble salt.

Two years later McCance & Widdowson (1942–3*a*) reported investigations on the absorption of calcium from bread. Their experiments were of particular interest because they were performed on human beings. They found that the absorption of calcium and magnesium decreased markedly when bread made from flour of 69% extraction was replaced by bread made from flour of 92% extraction, that is, when the content of phytic acid in the food was increased. They further found that an addition of phytic acid to white bread (69% extraction) caused a fall in the absorption of calcium that was so serious that the calcium balance in some subjects became negative. Finally it was shown that addition of $CaCO_3$ or $CaHPO_4$ improved the absorption of calcium considerably. In further experiments it was found that with diets containing respectively white bread, brown bread with partially split phytic acid and brown bread with the phytic acid intact the absorption of calcium decreased progressively in the order given.

It appears from all these researches that there is very good evidence that phytic acid in the food markedly prevents the absorption of calcium from the intestine of pigs and human beings, and that probably the same holds for magnesium.

Mellanby (1921, 1925) demonstrated that oatmeal has a marked rachitogenic effect on young dogs. Templin & Steenbock (1933) showed that the disappearance of this effect after boiling with acid was accompanied by a change of organic P to inorganic P in the cereals, and suggested that the organic P might not be as available as the inorganic. Bruce & Callow (1934) considered that the rachitogenic effect of cereals was due to their high content of phytic acid with phosphorus in a comparatively unavailable form. Harrison & Mellanby (1939) accepted the idea of the rachitogenic effect of phytic acid but laid stress on the calcium precipitating effect of this acid.

The influence of phytic acid upon the absorption of iron has been investigated by Widdowson & McCance (1942) in human beings. Brown bread with a high content of phytic acid seemed to depress the absorption of iron. As iron balances present

considerable technical difficulties, the two authors rightly regarded their results as suggestive rather than conclusive, and tried to obtain further information on the matter by new experiments in which the rise in the concentration of serum iron following a large dose of a soluble iron salt was taken as criterion of the absorption (McCance & Widdowson (1942–3*b*). In fourteen out of sixteen observations the addition of phytic acid prevented the full rise of the serum iron following the intake of the soluble iron salt. Taken together, these experiments support the opinion that phytic acid in the food has a depressing action on the absorption of iron from the intestine of man.

B. *New experiments.* In the course of investigations on rickets in pigs, performed in the laboratory of Møllgaard, it was established beyond all doubt that oats and maize have a very marked rachitogenic effect, whereas barley, wheat and wheat bran could be incorporated in the diet in large amounts without any such effect. As the phytic acid content of these grains is approximately the same (Table 1), it is evident that the suggestion of Bruce & Callow (1934) could not contain the whole truth. There must be some other difference in the composition of these grains responsible for their very unequal effect. As it was shown by Pedersen (1940) that the rachitogenic grains, oats and maize contained no phytase whereas this enzyme was found in varying amounts in the non-rachitogenic grains (Table 1), it was very obvious that this difference might provide the key to the understanding of the whole question of the rachitogenic effect of cereals. To investigate this possibility Pedersen made very extensive researches on pigs in the laboratory of Møllgaard. Having proved that the phytic acid in oats and maize could be split when the ground cereal was suspended under optimal conditions in a solution of phytase prepared by extracting wheat bran with saturated lime water, he proceeded to prepare such an extract on a scale sufficient for the treatment of the

Table 6. *Main results of experiments by Pedersen* (1940) *on the effect of phytic acid and phytase in the fodder on the development of rickets in groups of two pigs*

Group no.	State of phytic acid in fodder	State of phytase in fodder	Phytic P added as sodium phytate (g.)	Blood P (mg. %)	Degree of rickets
RI	Intact	Active	0	—	0
				—	0
RII	Intact	Inactive	0	—	+ +
				—	(+)
RIII	Split 50%	Inactive	0	—	+
				—	+
RVII	Intact	Inactive	2·5	3·47	+ +
				4·23	+ +
RVIII	Split	Inactive	2·5	7·88	0
				7·82	0
RIX	Intact	Active	2·5	5·18	0
				5·66	0
RX	Intact	Inactive	1·0	3·84	+ + +
				3·33	+ + +
RXI	Split	Inactive	1·0	7·40	(+)
				5·42	(+)
RXII	Intact	Inactive	0	4·55	+ +
				5·58	+
RXIII	Intact	Active	0	6·91	0
				7·12	0
RXV	Intact	Inactive	0*	5·27	(+)
				5·35	+ +
RXVI	Intact	Inactive	0†	7·25	0
				7·54	0

In the last column the degree of rickets is indicated as follows:

0 No macro- or microscopical rickets.
(+) Uncertain macro- or microscopical rickets.
\+ Slight macro- and microscopical rickets.
\+ + Severe macro- and microscopical rickets.
\+ + + Florid macro- and microscopical rickets.

* 15 g. $CaCO_3$ and 7000 i.u. vitamin D added daily.
† 12·5 g. $CaHPO_4.2H_2O$ and 7·5 g. $CaCO_3$ added daily.

whole ration of growing pigs. He prepared further pure crystalline sodium phytate in sufficient amounts to supply it to pigs as a source of phosphoric acid.

Groups of young pigs of 15–20 kg. live weight were then formed, each containing two animals. They received diets composed of different cereals with blood meal as a principal source of protein. Vitamins were partly provided by direct addition of concentrates (4000 i.u. of vitamin A, 200 i.u. vitamin B_1, 30 Sherman units of vitamin B_2 and 20 mg. ascorbic acid daily) and partly by 0·5 l. skim milk/kg. of dry food. The Ca content of the diet was in all experiments approximately 8 g./kg. of dry matter and the content of NaCl *c.* 6 g. The content of available phosphorus varied according to the amount of phytic acid in the food. For some groups the phytic acid was split before feeding by means of the treatment with phytase solution described above (indicated in Table 6 by 'split'). In some cases the whole food was heated before feeding to destroy the phytase (indicated by 'inactive'); in some, the phytase in the food was left intact and further increased by the addition of an extract of wheat bran (indicated by 'active'). Finally, in some groups, sodium phytate was directly added to the food. Each experiment lasted 6 days with a preliminary period of 8 days. The main results of these investigations are given in Table 6.

It will be seen from Table 6 that signs of rickets and a low phosphorus content of the blood are associated with the presence in the intestine of intact phytic acid. Rickets was absent or only very slight in the groups where the phytic acid had been split before feeding, or the animals received active phytase.

The results for groups RXV and RXVI show that even the addition of vitamin D cannot suppress entirely the rachitogenic effect of phytic acid but that the addition of $CaHPO_4$ prevents it completely.

It follows from these experiments that the phosphorus of phytic acid cannot be absorbed and participate in metabolism unless it is in some way split into free phosphoric acid and inositol, and this cannot take place in the intestine of the higher animals unless the food itself contains a certain amount of active phytase.

This conclusion was further substantiated by Pedersen's observation that, when the phytic acid was not split before feeding or the food contained no active phytase, the faecal phosphorus was very nearly equal to the phytic phosphorus in the food. If, on the other hand, the phytic acid was split, or the animal was fed active phytase, the faecal phosphorus was always less than the phytic phosphorus in the food.

Man commonly eats food with the phytase activity entirely destroyed by heating, and there seems to be no possibility of any cleavage of phytic acid in his intestinal canal. The rachitogenic effect of phytic acid must therefore be more serious for man than for the pig unless human food is, in some way or another, supplied with inorganic phosphate in sufficient amounts to prevent the fall in absorption.

V. EXPERIMENTAL EVIDENCE OF THE BENEFICIAL EFFECT OF ORGANIC OXY-ACIDS ON THE ABSORPTION OF CALCIUM AND PHOSPHORUS FROM RATIONS CONTAINING PHYTIC ACID

It has been mentioned that an addition of oxy-acids to the food or a considerable production of lactic acid in the intestinal canal of mammals might prevent the harmful effect of phytic acid on the absorption of calcium and phosphorus.

Evidence of such effect is found in the investigations of Møllgaard (1945) on the effects of oxy-acids on pigs. Four pigs, all females, weighing 20–23 kg., were used for these experiments. Their food was made up of feeding stuffs commonly used for raising young pigs in Denmark, namely barley, wheat, wheat bran, blood meal and lucerne meal. Sufficient $CaHPO_4$ and $CaCO_3$ were added to provide, together with the calcium and phosphorus already present, a total supply of 9 g. Ca and 6 g. P/kg. dry matter; the animals were given to drink, with each kg. dry matter, 500 g. of skim milk. About 60 % of the total phosphorus was present as phytic acid. The phytase activity of the grains and bran was not destroyed before feeding, so that the animals had the chance to split the phytic acid during digestion. The effect of oxy-acids on the absorption of calcium and phosphorus was studied under such relatively favourable conditions. Tartaric and lactic acids were used and the pigs were divided into two groups. In the first period of the experiment with tartaric acid pigs 43 and 45 received daily 13·5 g. of the acid and pigs 47 and 48 none. In the second period pigs 47 and 48 received 16·5 g. of the acid, and pigs 43 and 45 served as controls. The experiment with lactic acid was similarly arranged. Each experimental period lasted 6 days with a preliminary period of 7–8 days. The results are given in Table 7.

The results show that the two oxy-acids caused a considerable increase in the absorption of phosphorus and particularly of calcium.

Experiments were also performed with diets containing only 4 g. Ca/kg. dry matter (Table 8).

This time the percentage absorption was naturally somewhat higher in all groups, but the effect of the oxy-acid was again obvious.

Table 7. *Effect of oxy-acids on the absorption of* P *and* Ca *by the pig*

No. of animal and period	Intake (g./day) P	Intake (g./day) Ca	Percentage absorption P	Percentage absorption Ca	Oxy-acids added to food (g./day)
43_{I}	5·62	7·64	42·3	43·2	Tartaric acid 13·5
45_{I}	5·62	7·64	42·3	46·3	,, ,, 13·5
47_{II}	6·90	9·29	43·6	35·6	,, ,, 16·5
48_{II}	6·90	9·29	44·1	38·6	,, ,, 16·5
43_{III}	8·16	10·97	41·2	42·3	Lactic acid 8·0
45_{III}	8·16	10·97	37·5	39·7	,, ,, 8·0
47_{IV}	9·41	12·66	43·1	41·9	,, ,, 8·8
48_{IV}	9·41	12·66	37·1	39·3	,, ,, 8·8
47_{VI}	10·81	14·31	38·4	37·3	Lactic acid 20·0
48_{VI}	10·81	14·31	39·3	40·3	,, ,, 20·0
47_{I}	5·62	7·64	28·3	30·8	Controls for tartaric acid
48_{I}	5·62	7·64	34·7	38·5	
43_{II}	6·90	9·29	35·2	34·2	
45_{II}	6·90	9·29	36·4	26·4	
47_{III}	8·16	10·97	37·7	36·2	Controls for lactic acid
48_{III}	8·16	10·97	33·0	34·6	
45_{IV}	9·14	12·66	36·1	35·8	
47_{V}	10·21	13·49	39·2	34·7	Controls for lactic acid
48_{V}	10·21	13·49	37·2	32·5	
45_{VI}	10·81	14·31	36·0	32·9	
Mean of all experiments with oxy-acids in the food			40·6	40·2	
Mean of all experiments without oxy-acids in food			37·3	33·8	

Table 8. *Effect of tartaric acid on the absorption of* P *and* Ca *by the pig from a diet relatively low in calcium*

No. of animal and period	Intake (g./day) P	Intake (g./day) Ca	Percentage absorption P	Percentage absorption Ca	Oxy-acid added to food (g./day)
45_{VII}	9·05	5·94	56·1	48·0	25·5 g. tartaric acid
47_{VIII}	9·05	5·94	54·5	51·5	
48_{VIII}	9·05	5·94	46·3	42·9	
Mean			52·3	47·5	
47_{VII}	9·05	5·94	45·2	38·2	No oxy-acid
48_{VII}	9·05	5·94	41·9	34·7	
45_{VIII}	9·05	5·94	46·5	45·3	
Mean			44·5	39·4	

In the experiments described so far no vitamin D was given to the pigs. Table 9 contains results of tests done at the same time with two other pigs which received 1200 i.u. daily of vitamin D but no oxy-acids.

It will be seen that the effect of vitamin D was somewhat greater than that of oxy-acids in the experiments where the calcium content of the food was high, but was of the same order of magnitude in the experiments with food lower in calcium, a fact that underlines the importance of these acids in the absorption of calcium and phosphorus.

Table 9. *Effect of vitamin D added to the food on the absorption of* P *and* Ca *by the pig*

No. of animal and period	Intake (g./day) P	Intake (g./day) Ca	Percentage absorption P	Percentage absorption Ca	Vitamin D (i.u./day)
86_{I}	5·47	6·71	45·5	39·8	1200
87_{I}	5·47	6·71	44·6	41·1	1200
86_{II}	6·36	7·79	49·8	47·4	1200
87_{II}	6·36	7·79	41·5	41·5	1200
86_{III}	7·59	10·14	50·1	50·0	1200
87_{III}	7·59	10·14	43·1	43·1	1200
86_{IV}	8·50	10·37	55·8	56·6	1200
87_{IV}	8·50	10·37	47·5	47·8	1200
Mean			47·2	45·9	

At the conclusion of these experiments tubes were inserted in the jejunum of pigs 45 and 48 and in the ileum of pig 47 for the collection of intestinal contents from which it was proposed to cultivate lactic acid bacilli in order to get a culture that would multiply in the intestine of the pig. By adding such culture to the food it should be possible to increase the concentration of lactic acid in the intestinal contents. Success was achieved with pig no. 48. The isolated bacillus formed, when grown on sterilized skim milk, large amounts of lactic acid, but at temperatures between 30 and 40° only minute amounts of volatile fatty acids. The three pigs were fed ordinary skim milk in a preliminary period and after that they received the same amount of skim milk inoculated with the culture and left at 39° for 24 hr. In both periods samples were taken from intestinal tubes 3 hr. after feeding, and the lactic acid content was determined by a modified method of Flieg (1937). The results are seen in Table 10.

Table 10. *Effect of acidified skim milk on the concentration of lactic acid in the contents of the small intestine of pigs*

Pig no.	(m-equiv. lactic acid/kg. contents) Preliminary period	Experimental period	Percentage increase in lactic acid	Skim milk fed (kg.)
45	75	119	58·7	0·5
47	134	168	25·4	0·5
48_{I}	90	104	15·6	0·5
48_{II}	90	131	45·5	0·5

As the culture increased the production of lactic acid in the pigs' intestine, a large-scale experiment with pigs was set up in order to investigate whether these animals would grow better when fed skim milk inoculated with this culture in addition to an ordinary ration containing phytic acid phosphorus in amounts of 60 to 66% of the total phosphorus. The outcome of this first experiment was that the

growth of the group which received the inoculated milk was distinctly superior to the growth of the control group. Because of the importance of the problem in practical feeding the experiment will be repeated, but the preliminary results already suggest that lactic acid fermentation is of advantage to growth and thereby support the view that the elimination of the harmful effects of phytic acid is of real importance to the metabolism of the pig.

VI. THE NEED FOR INCREASING THE ABSORPTION OF CALCIUM, PHOSPHORUS AND IRON FROM THE ORDINARY FOODSTUFFS USED FOR HUMAN CONSUMPTION

The experiments described in §§ IV and V afford good reason for the conclusion that the theoretical assumptions discussed in § III were correct. The presence of considerable amounts of phytic acid in the food must be considered as harmful to the absorption of calcium, phosphorus and iron. The harmful effect can be prevented either by splitting the phytic acid in the food or by adding sufficient amounts of calcium phosphate and of iron salts to the food.

In animals the cleavage of phytic acid during digestion may to some extent be brought about by the phytase present in some of the feeding stuffs, and it can be further enhanced by an increase in the production of lactic acid in the intestine. In human beings this is seldom possible because their food is mostly heated to such temperature that any enzymic activity is destroyed. There are, therefore, only two possibilities of preventing the harmful effect of phytic acid in human food: the cleavage of the acid before consumption, and the addition to the food of suitable amounts of calcium phosphate and of iron salts. This can, of course, be done by incorporating in the ration other foodstuffs rich in calcium, phosphorus and iron, but containing little or no phytic acid. In modern industrial society, however, the bulk of the population of any country has not access to such foodstuffs. It consumes mostly foods which are produced in large amounts and at relatively cheap prices by the food industry. It is therefore of decisive importance for the well-being and ability to work of these communities that foodstuffs should reach consumption in such a state that they supply the consumers with the amino-acids, vitamins and minerals necessary for nutritional welfare and do not contain any substance that in some way or another may be harmful to metabolism.

With the purpose of improving in this respect the daily bread of the Danish population, work was done in the Physiological Laboratory of the Agricultural Experimental Station and the Central Laboratory of the Co-operative Bakeries in Denmark. It concerns for the present the possibility of increasing the calcium content and of splitting the phytic acid of rye bread so extensively consumed by the Danish population. Further work on the supply of iron and vitamins will, however, follow.

VII. THE ADDITION OF CALCIUM TO BREAD

The necessity to increase the calcium content of the Danish common rye bread is made obvious by Table 11 which lists Danish foodstuffs containing appreciable quantities of calcium. Out of seventy-four Danish foodstuffs analyzed we found only fourteen which could serve as a source of calcium and of these only nine contained considerable quantities.

Table 11. *The calcium content of some Danish foodstuffs*

1% and more		0·5–1·0%		0·1–0·5%	
Spinach	2	Cucumber	0·8	Carrots*	0·3
Lettuce	1	Green beans	0·6	Leeks	0·4
Kale*	2	White cabbage*	0·9	Cauliflower	0·2
Milk	1	Brussels-sprouts*	0·5	Tomatoes	0·15
Cheese	1·5			Fish*	0·1–0·17

* Generally accessible.

Of these foodstuffs only five, marked with an asterisk in the table, are available at prices within the reach of the general population in the towns of Denmark. The table shows that the diet of the ordinary town population in Denmark must be exceedingly poor in calcium. The prevalence of dental diseases in this country bears witness to the harmful effect of this condition.

It was therefore agreed that approximately 0·5% of calcium should be added to the rye bread produced by all the forty-one bakeries belonging to the Workers' Co-operative in Denmark. This was

Table 12. *Calcium content of bread from* 30 *bakeries*

Bakery	Ca in dry matter (%)	Bakery	Ca in dry matter (%)
Aalborg	0·54–0·40	Nyborg	0·37
Aarhus	0·54–0·33	Nykøbing F	0·60
Assens	0·46	Nykøbing M	0·56
Esbjerg	0·30–0·36	Nærum	0·36–0·53
Fredericia	0·24–0·20	Randers	0·41
Frederikshavn	0·49	Ringsted	0·45–0·47
Grenaa	0·57–0·51	Roskilde	0·40–0·60
Glostrup	0·48–0·44	Rønne	0·30–0·46
Haderslev	0·53	Skive	0·47
Helsingør	0·45–0·51	Slagelse	0·41–0·57
Holbæk	0·46	Svendborg	0·48–0·50
Kalundborg	0·46–0·43	Thisted	0·54
Køge	0·41–0·42	Vejle	0·51
København	0·47–0·60	Viborg	0·38–0·58
Nakskov	0·42	Vordingborg	0·46–0·70

done by adding 1% of $CaCO_3$ to the flour. The calcium content of the bread manufactured by the Co-operative in September 1945 is given in Table 12.

It appears from the table, that the bread from the great majority of bakeries contained 0·4 to 0·6% of calcium, which should be satisfactory.

VIII. THE SPLITTING OF PHYTIC ACID IN THE DOUGH

The splitting of the phytic acid in the dough is a much more difficult undertaking than the addition of calcium, particularly because this addition very naturally delays the splitting by precipitating the acid as insoluble calcium phytate.

In our work we have, however, taken advantage of the fact that oxy-acids are able to keep the calcium and the phytate ions in solution. It is shown in Fig. 2 that tartaric acid is able to dissolve the ions even in alkaline solution. Lactic acid, of course, is not so effective in concentrations which are possible in the dough, but it can work within the pH interval 4·5–5·5 and this is quite sufficient, because the optimal pH for the action of phytase is approximately 5. We used tartaric acid for the study of the fundamental conditions of cleavage, but in the practical application of the method in the bakeries we have made use of the lactic acid produced in the dough by fermentation. In all experiments in the laboratory and in bakeries the rye flour was of 98% extraction.

A. *The effect of pH on the cleavage of phytic acid in the dough.* To 100 g. of rye flour containing 0·5% Ca was added 1 g. of tartaric acid, and the pH was brought to the values quoted in Table 13 by adding water and suitable amounts of HCl and NaOH, the total amount of fluid added being 80 ml. The dough was left at a temperature of 28–30° for 4 hr. The results are given in Table 13.

Table 13. *The effect of pH on the cleavage of phytic acid in rye dough during incubation in the presence of tartaric acid*

pH	Moisture in dough (%)	Total P in dry matter (%)	Phytic P in dry matter: Initial (%)	Phytic P in dry matter: After 4 hr. (%)	Extent of hydrolysis (%)
4·60	47·4	0·510	0·383	0·167	56·4
4·95	47·7			0·109	71·5
5·25	48·2			0·065	83·0
5·47	48·7			0·082	78·6
5·70	48·2			0·071	81·5
5·93	48·1			0·081	78·9

It appears from the table that there is an optimum at pH 5·3, but after this the curve flattens out, so that the difference in action between pH 5·3 and 5·9 is not marked.

B. *The effect of the water content on the cleavage of phytic acid in the dough.* To 100 g. of rye flour containing 0·5% Ca was added 1 g. of tartaric acid. The pH was brought to 5·5–5·6 and varying amounts of water were added to the dough which was left at a temperature of 28–30° for 4 hr. The results appear in Table 14.

Table 14. *The effect of the water content on the cleavage of phytic acid in rye dough during incubation in the presence of tartaric acid*

Moisture content (%)	pH	Total P in dry matter (%)	Phytic P in dry matter: Initial (%)	Phytic P in dry matter: After 4 hr. (%)	Extent of hydrolysis (%)
49·1	5·5	0·510	0·383	0·043	88·8
46·9	5·5			0·064	83·3
44·5	5·6			0·090	76·5
41·3	5·5			0·111	71·0

The table shows that the water content of the dough has a considerable effect on the velocity of cleavage. To reach a maximum in 4 hr. the water content should be increased to 48–49%.

C. *The effect of temperature on the cleavage of phytic acid in the dough.* To 100 g. of rye flour with 0·5% Ca were added 1 g. of tartaric acid and 82 ml. water. The pH was stabilized at 5·3 and the dough left at the temperatures indicated in Table 15 for 4 hr.

The table shows that the rate of hydrolysis increases with increasing temperature, but that it seems not to have reached maximum even at 39°. Table 16 shows that it is a question of the concentration of the oxy-acid.

D. *The effect of the concentration of the oxy-acid on the cleavage of phytic acid in the dough.* To 100 g. of rye flour with 0·5% Ca were added the amounts of

Table 15. *The effect of temperature on the cleavage of phytic acid in rye dough during incubation in the presence of tartaric acid*

Temperature (°)	pH	Moisture content (%)	Total P in dry matter (%)	Phytic P in dry matter: Initial (%)	Phytic P in dry matter: After 4 hr. (%)	Extent of hydrolysis (%)
10–12	5·3	50·7	0·536	0·409	0·264	35·5
18–20		50·4			0·180	56·0
25–27		50·2			0·120	70·7
39		50·1			0·066	83·7

Table 16. *Effect of concentration of tartaric acid on the cleavage of phytic acid in rye dough during incubation at* 28–30°

Tartaric acid (g./100 g. flour)	pH	Moisture content (%)	Total P in dry matter (%)	Phytic P in dry matter: Initial (%)	Phytic P in dry matter: After 4 hr. (%)	Extent of hydrolysis (%)
1·6	5·7	48·5			0·011	96·9
0·8	6·0	49·0	0·489	0·361	0·083	77·0
0·4	6·15	48·5			0·129	64·3
1·5*	5·3	50·0	0·536	0·409	0·082	80·0

* Temperature of incubation 25–27°.

tartaric acid shown in Table 16. The pH was brought to 5·7–6·1 and 80 ml. water added. The dough was kept at 28–30° for 4 hr.

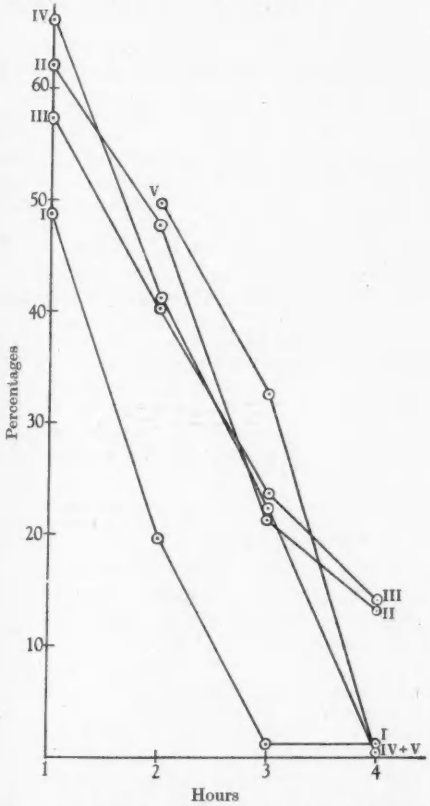

Fig. 4. The effect of variation in time of reaction and in concentration of Ca and of tartaric acid on the cleavage of phytic acid. Curve I. 1% tartaric acid + 0·1% Ca. Curve II. 1% tartaric acid + 0·17% Ca. Curve III. 1% tartaric acid + 0·5% Ca. Curve IV. 1·5% tartaric acid + 0·5% Ca. Curve V. 1·6% tartaric acid + 0·5% Ca. The abscissa indicates the reaction time in hours, the ordinate represents phytic P in percentages of phytic P at the beginning of experiment.

It appears from the table that the rate of hydrolysis increases with the concentration of the oxy-acid until maximum is reached at 1·6%. It is further shown that, with this concentration, phytic acid is largely split even at a temperature of 25–27°.

E. *The effect of variation in time of reaction and concentration of* Ca *and of oxy-acid.* Fig. 4 shows the influence of variation in the time of reaction and in concentration of Ca and of tartaric acid.

From the curves in Fig. 4 the following facts are evident.

(1) The velocity of cleavage of phytic acid is clearly dependent on the quantitative ratio of Ca to tartaric acid.

(2) The degree of splitting depends on the time of reaction.

F. *Summary of results.* From all these experiments the optimal condition for the enzymic cleavage of phytic acid in the rye dough can be stated. They are as follows:

Water content	48–49%
pH	5·1–5·3
Tartaric acid content	1·5%
Temperature of reaction	28–30°
Time of reaction	4 hr.

Further experiments have shown that these conditions are applicable only to fresh flour. If the flour has been kept for a long time at room temperature (e.g. 1 month), the phytase content evidently decreases and the degree of splitting after 4 hr. is diminished.

IX. INFLUENCE OF THE BAKING PROCESS ON THE SPLITTING OF PHYTIC ACID

The investigations just described have established the optimal conditions for the splitting of phytic acid in the dough. They have not taken account, however, of the possible effect of the baking process itself. This was done in the experiments summarized in Table 17.

In all these experiments the dough was acidified by the culture of the lactic acid bacillus mentioned on p. 596. In some of the experiments 1·5% tartaric acid was also added to the dough.

Table 17. *Effect of baking on the phytic acid content of rye bread*

(Fermentation time, i.e. time interval between final preparation of dough and placing in oven, 4 hr. Fermentation temperature 28–30°. Ca content of flour 0·5%.)

	Addition to dough	pH	Moisture (%)	Total P in dry matter (%)	Phytic P in dry matter (%)	Phytic P (as % of total P)
Dough	Tartaric acid + culture of	4·61	44·2	0·523	0·125	23·90
Bread	lactic acid bacillus	4·75	38·5	0·523	0·055	10·52
Dough	Tartaric acid + culture of	4·82	44·82	0·507	0·105	20·71
Bread	lactic acid bacillus	5·20	39·19	0·507	0·035	6·90
Dough	Culture of lactic acid	4·98	45·83	0·517	0·120	23·21
Bread	bacillus alone	5·20	38·76	0·517	0·061	11·80
Dough	Tartaric acid + culture of	4·86	46·56	0·509	0·086	16·89
Bread	lactic acid bacillus	5·16	40·22	0·509	0·035	6·88
Dough	Culture of lactic acid	4·92	47·25	0·509	0·089	17·48
Bread	bacillus alone	5·08	40·44	0·509	0·054	10·61

It will be seen that the baking process invariably causes a considerable decrease in phytic acid P. This means that the splitting is continued and probably even accelerated by the baking process. This result is very naturally explained by the fact that the temperature in the bread increases very slowly up to about 100° during baking. As the phytase is not destroyed until the temperature reaches about 70°, there is always time for it to act.

The table shows further that the cleavage of phytic acid in the dough prepared with the culture alone was only slightly less than that obtained with added tartaric acid. This indicates that under practical conditions of baking the splitting of phytic acid might probably be effected by a strong lactic acid fermentation alone. That this can be done is proved by the experiments described in Table 18.

The acidifier in these experiments was the lactobacillus mentioned on p. 596. The flour contained in all cases 0·5% Ca.

To understand the results it must be kept in mind that the oven in the Central Laboratory is of relatively small size compared with the big ovens in the bakeries. In a small electrically heated oven the temperature rises rapidly to maximum which means that the phytase is destroyed within a short time after the breads are put in the oven. In the big ovens, on the contrary, the temperature increases slowly and the phytase has a considerable time to do its work.

For this reason the influence of the time of holding the dough before baking must be more pronounced with the small oven. The table shows clearly that this is the case. In the big bakeries there was little difference in the extent to which the phytic acid was split after holding times of 1 and

Table 18. *The phytic acid content of rye bread baked after lactic acid fermentation of the dough*

(Fermentation temperature 28–31°. Ca content of flour 0·5%.)

Bakery	Date	Fermentation time* (hr.)	pH	Moisture (%)	Total P in dry matter (%)	Phytic P (as % of total P)
Central Laboratory	8. xi	1	5·70	37·0	0·449	30·51
Central Laboratory	8. xi	1	5·63	39·1	0·475	28·42
Glostrup	8. xi	1	5·21	37·2	0·404	19·18
Central Laboratory	9. xi	2	5·22	36·4	0·493	16·84
Central Laboratory	9. xi	2	5·22	39·7	0·524	16·79
Central Laboratory	10. xi	3	5·17	37·2	0·511	10·96
Central Laboratory	10. xi	3	5·19	37·8	0·528	8·14
Slagelse	8. xi	2	4·81	36·2	0·475	9·47
Slagelse	9. xii	1·75	5·96	37·8	0·375	17·07
Helsingør	15. xi	1·5	5·32	36·5	0·406	17·98
Helsingør	15. xi	1·5	5·26	37·2	0·404	18·07
Aarhus	15. xi	2	—	35·5	0·456	15·35
Glostrup	20. xi	1	5·11	39·3	0·422	18·72
Glostrup	20. xi	1	5·17	38·1	0·425	18·59
Viborg	6. xii	2	4·90	—	0·484	18·80
Roskilde	19. xii	1·75	4·86	36·9	0·421	15·68
Rønne	19. xii	2·5	4·90	40·4	0·502	17·93

* The fermentation time indicates the time interval between final preparation of dough and placing in oven.

2 hr. and the variation in moisture content did not greatly affect the results. This meant that big bakeries should have no difficulty in splitting phytic acid to such an extent that bread would contain 82 to 85 % of its total phosphoric acid in a state available for absorption.

After these results had been obtained it was decided that all the bakeries belonging to the Co-operative should as soon as possible change their method of baking according to the following rules:

(1) The dough should be acidified with the lactobacillus mentioned on p. 596. The cultures should be delivered from the Central Laboratory in order to ensure uniformity.

(2) The pH in the bread should be kept at 5·0–5·3.

(3) The moisture content of the dough should be increased to 46–48 %.

(4) The fermentation time should be about 2 hr. After one year's trial the results from thirty Co-operative bakeries were as set out in Table 19.

Table 19. *Available phosphorus in rye bread baked by Co-operative bakeries according to the rules mentioned above*

Bakery	Non-phytic P (as % of total P)			Bakery	Non-phytic P (as % of total P)		
Aalborg			84	Nyborg			83
Aarhus		81	85	Nykøbing F			80
Assens			90	Nykøbing M			79
Esbjerg		87	91	Nærum		81	68
Fredericia		82	80	Randers			81
Frederikshavn			79	Ringsted		84	84
Grenaa			79	Roskilde	83	84	83
Glostrup	79	81	81	Rønne		84	82
Haderslev			81	Skive			77
Helsingør	83	82	96	Slagelse	84	84	79
Holbæk			87	Svendborg		83	83
Kalundborg		84	85	Thisted			82
Køge		82	88	Vejle			80
København	75	77	67	Viborg		87	81
Nakskov			73	Vordingborg		82	90

The table shows that the percentage of non-phytic P in the bread from twenty-three of the thirty bakeries was 80–85. For six of them the percentage lay between 75 and 80; one only had a percentage under 75.

These results seem to be satisfactory from a nutritional point of view. Of course the phytic acid ought to be split entirely, but this goal is probably unattainable as long as $CaCO_3$ must be used for the enrichment of the bread with Ca, because calcium lactate itself delays the cleavage to a certain degree. The conditions will be much more favourable as soon as it is possible to import $CaHPO_4$ in sufficient amounts to supply our bread with 0·5 % of Ca, because this salt has only a very slight influence on the splitting process. This is seen from Table 20.

It appears that the delaying effect of $CaHPO_4$ is very small whether lactic or tartaric acid is used. It

Table 20. *The effect of* $CaHPO_4$ *on the cleavage of phytic acid in rye dough*

(Temperature of fermentation 27–29°. Fermentation time 4 hr.)

pH	Lactic acid (%)	$CaHPO_4$ added (%)	Non-phytic P (as % of phytic P in the flour)
5·3	*c.* 1·0	0	80·13
5·1	*c.* 1·0	1·7	80·76
	Tartaric acid (%)		
5·0	*c.* 1·0	0	89·79
5·1	*c.* 1·0	1·7	85·13

is therefore the intention of the Central Laboratory to supply the bakeries with $CaHPO_4$ instead of $CaCO_3$, as soon as this salt is available.

X. THE STATE OF PHOSPHORIC ACID IN THE BREAD AFTER THE CLEAVAGE OF PHYTIC ACID

So far the question of the state of the phosphoric acid after cleavage had not been taken into consideration. To find out whether it is all present as phosphoric ions or partly in organic combination, the analyses reported in Table 21 have been carried out. The table shows the analytical distribution of the phosphoric acid after almost complete cleavage of the phytic acid in the dough. The first half of the table contains the analyses of the flour used for making the dough and the second half the analyses of the dough after splitting. The term inorganic P means the amount of P directly precipitated by ammonium molybdate in an extract of the dough with 0·5N-HCl. The term residual P means the difference between the total P and the sum of inorganic P and phytic P.

Table 21. *The distribution of various forms of phosphorus in rye flour and in dough prepared from the flour*

(Ca-content in Exp. 1, 0·1 %; in Exps. 2 and 3, 0·5 %.)

Exp.	Phytic P (as % of total P)	Inorganic P (as % of total P)	Residual P (as % of total P)	Tartaric acid added to flour (%)
		Rye flour		
1	74·7	14·3	11·0	1·0
2	75·9	11·1	13·0	1·5
3	75·1	10·0	14·9	1·6
		Dough		
1	0·8	69·7	29·5	
2	0·4	63·9	35·7	
3	0·4	62·0	37·6	

It appears from the table that the residual P increases during the cleavage of the phytic acid. This means that the total phosphoric acid even after complete cleavage is not present as phosphoric ions, but that some of it is in fact bound to organic substances.

Table 22. *Distribution of various forms of phosphorus in rye bread*

Bread no.	pH	Moisture (%)	Total P in dry matter (%)	Phytic P (as % of total P)	Inorganic P (as % of total P)	Residual P: Extractable* (as % of total P)	Residual P: Non-extractable* (as % of total P)
94	5·17	37·18	0·511	10·96	65·36	18·79	4·89
95	5·19	39·79	0·538	8·14	64·77	19·89	7·20
97	5·10	35·47	0·456	15·35	62·06	16·67	5·92
100	5·32	36·50	0·406	17·98	59·11	14·54	8·37
102	5·10	39·33	0·422	18·72	61·61	15·64	4·03

* With 0·5 N-HCl.

In order to elucidate the nature of these phosphoric compounds we have further determined how much of the residual P is soluble in 0·5 N-hydrochloric acid. This is seen in Table 22, which presents the analytical distribution of the phosphorus after cleavage in the bakeries.

The table shows that a certain fraction of the residual P cannot be dissolved in 0·5 N-HCl. This is probably identical with the phosphorus contained in the proteins of the flour and is, according to present knowledge, set free during digestion in the intestinal canal. The extractable residual P, however, may be, at least partly, made up of mono- and diphosphoric esters of inositol, which are not measured in the analytical determination of phytic acid, because they are not precipitated by iron. It is more probable that this fraction results from phosphorylation during the fermentation of the dough in rising. It must be kept in mind that under practical conditions a certain amount of yeast is added to the dough to effect rising, and in the fermentation with yeast processes of phosphorylation of carbohydrates play, as is well known, a considerable role. If that is true, the extractable residual P would also be available for absorption in the intestines. The question has not yet been definitely settled, but it seems reasonable to assume that, at all events, a large part of the extractable residual P is available in digestion.

SUMMARY

1. In grains used for human consumption and for feeding farm animals 75 to 85% of the total phosphorus is found in phytic acid.

2. The rachitogenic effect of cereals depends on the presence in them of phytic acid. Experimental evidence is given to show that when this is hydrolyzed the rachitogenic effect disappears.

3. The rachitogenic grains, oats and maize, contain no phytase whereas considerable amounts of this enzyme are present in the non-rachitogenic grains, wheat, rye and barley. With these grains an enzymic cleavage of phytic acid may take place in the intestine of animals.

4. Phytic acid forms a calcium salt insoluble at a pH of 3–4, which means that phytic acid may precipitate Ca even in the first parts of the small intestine, thus causing a serious fall in its absorption. Experiments, which prove this harmful effect, are described.

5. Oxy-acids, such as tartaric, citric and lactic acids, are shown to shift the precipitation point of calcium phytate to a more alkaline reaction. It is demonstrated that these oxy-acids considerably improve the absorption of Ca and P from the intestine of pigs.

6. It is shown that the feeding to pigs of a culture of a lactobacillus which can grow in their intestine increases the amount of lactic acid formed during digestion. Pigs fed such cultures grow better than controls not receiving the culture.

7. Rye bread in Denmark is improved by the addition of 0·5% of Ca and hydrolysis of 80 to 85% of the phytic acid by enzymic fermentation during the preparation of the dough and the first stages of baking. Analyses of the bread from 30 bakeries belonging to Co-operative Societies in Denmark are published.

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Ox-spleen β-Glucuronidase; its Purification and a Study of some Factors Involved in Assaying its Activity

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(*Received* 15 *April* 1946)

Masamune (1934) showed that aqueous extracts of ox kidney contained an enzyme which appeared to be specific in catalyzing the hydrolysis of β-glucuronides at the glucosidic link. The method of purification developed by Masamune for this 'β-glucuronidase' involved autolysis of the kidney with water and precipitation of the enzyme with ethanol. Oshima (1936) carried the purification a stage further than Masamune by adsorbing the enzyme on kaolin and subsequently eluting it with sodium phosphate. In repeating the work of the Japanese investigators Fishman (1939*a*, *b*) found difficulty in obtaining active enzyme preparations and devised an entirely new method for its purification from ox spleen. In this method acetone precipitation of the enzyme from the aqueous spleen extract was followed by an isoelectric precipitation of inactive protein. The enzyme preparation was further purified by fractionation with various concentrations of ammonium sulphate.

In view of the suggestion by Fishman (1940) that β-glucuronidase might catalyze the synthesis of conjugated glucuronides in the animal body, its chemical behaviour merits further study. The present research was therefore initiated with the ultimate intention of carrying out a systematic physicochemical examination of β-glucuronidase action. During the preliminary work Fishman's method (1939*a*) of purifying the enzyme from ox spleen was repeated. Since it was found that a large loss in activity occurred as a result of the acetone precipitation a different method was devised and is described below. Some factors involved in the estimation of β-glucuronidase activity in spleen extracts were investigated and are also described.

EXPERIMENTAL

The estimation of β-glucuronidase activity

Bio-synthetic *l*-menthol glucuronide prepared by the method of Williams (1938) was crystallized three times from water. The substrate solution was prepared by suspending 500 mg. *l*-menthol glucuronide ($C_{16}H_{28}O_7 \cdot 1{\cdot}5H_2O$) in about 10 ml. water. A few drops of N-NaOH were added and the mixture was warmed to effect solution. After cooling it was titrated electrometrically to pH 5·0 with N-NaOH and diluted to 25 ml.

Enzyme (0·50 ml.) was added to acetate buffer (0·10M, 1·0 ml., pH 5·0) and substrate (0·50 ml.) in a 15 ml. pyrex centrifuge tube which was stoppered with a rubber bung and incubated for 2 hr. at 25°. The protein was then precipitated, centrifuged down, and the reducing power of a suitable measured sample of supernatant liquid was determined by Levvy's modification (1946) of the cerimetric method. A control tube in which enzyme solution, buffer and water were placed in the same proportion as above was treated in an identical manner. Since the ceric sulphate was standardized against glucurone the amount of reducing material found in each tube was calculated in terms of glucuronic acid by applying a correction factor. From the difference between the two estimations the amount of glucuronic acid liberated by 1 ml. of enzyme solution was calculated.

Under the conditions of the activity measurement it was found that the time-action curve was of zero order. If the concentration of the enzyme solution used for assay was adjusted so that the amount of substrate hydrolyzed was less than 10%, the rate of hydrolysis was directly proportional to the enzyme concentration. It is therefore proposed to define one β-glucuronidase unit (G.U.) as that amount of enzyme which will liberate 0·100 mg. of glucuronic acid from the substrate *l*-menthol glucuronide under the conditions specified.

Liberation of reducing substance from ox-spleen extract

During the preliminary work on the purification of the enzyme, Fishman's practice (1939*a*) of controlling the enzyme assay by adding substrate to boiled enzyme was followed. However, under the conditions for estimating enzyme action in the present investigation, the amount of substrate hydrolyzed was rather small and results obtained for β-glucuronidase activity of a spleen extract on several successive days were often erratic. It soon appeared that this type of control was unsuitable since dialyzed aqueous extracts of acetone-treated spleen (described in detail later, stage A, Table 1), when incubated alone formed a relatively large amount of reducing substance which was estimated in the ceric sulphate titration.

Enzyme solution previously heated to 100° for 3 min. did not liberate any reducing material on incubation at 25°. At any time during incubation of the enzyme extract at 25°, the reducing material which had been formed was not destroyed by heating for 3 min. at 100°. In most cases the amount of reducing material formed during incubation of the enzyme at 25° was many times greater than the amount of glucuronic acid formed when substrate was present. Consequently little reliance could be placed on the figure obtained for β-glucuronidase activity of the spleen extracts. One stage of purification did, however, remove a large proportion of the precursor of this reducing substance; the error consequently was most serious in the assay of the initial aqueous extract.

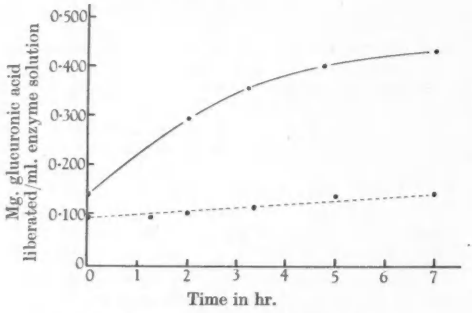

Fig. 1. Liberation of reducing material from aqueous extract of acetone-treated ox spleen by incubation at 25°. Solid line: spleen extract; broken line: after dialysis of extract incubated at 25° for 7 hr.

The solid line in Fig. 1 shows the time-action curve for liberation of reducing material by a spleen extract (A, Table 1) over a 7 hr. period at 25° and pH 5·0. The rate of release of reducing material during reincubation, after 24 hr. dialysis, is represented by the broken line. (The reducing substance was treated as if it were glucuronic acid and is plotted as such in Fig. 1.)

It is seen that the reducing material formed during the incubation period was removed by dialysis and that there was little further liberation of this material on subsequent incubation at 25°. Similar results were obtained with several different spleen extracts. In some cases the production of reducing material was virtually complete after 5 hr. incubation, in others as much as 8 hr. were required for completion of the process. A method was thus furnished for avoiding the high blank that would otherwise be obtained in assaying the activity of the initial spleen extract. When spleen was not subjected to the preliminary acetone treatment, the amount of reducing material formed on incubation of an aqueous extract was much greater than in extracts prepared after acetone treatment of the tissue.

Purification of β-glucuronidase

Throughout the procedure pH was controlled with a glass electrode. At each step in the purification enzyme activity was determined. Protein nitrogen (P.N.) was precipitated from samples of enzyme solution by addition of an equal volume of 10% trichloroacetic acid (w/v) and estimated by the micro method of Ma & Zuazaga (1942). The ratio G.U./mg. P.N. for an enzyme solution has been used as a basis for calculating purity after each stage of the purification. Figures for activity and P.N. at each stage in the purification are given in Table 1 for a representative preparation.

Table 1. *Purification of β-glucuronidase; activity, protein nitrogen and purity at various stages of a representative preparation*

Enzyme solution: Stage of purification	Volume (ml.)	Activity/ml. (G.U.)*	Total activity (G.U.)*	Protein-N (mg./ml.)	Activity/mg. protein-N (G.U.)*
A	2,365	0·61	1,432	2·16	0·28
B	2,365	1·27	3,050	1·81	0·70
C	2,440	1·21	2,960	0·58	2·10
D	243	10·5	2,550	3·45	3·04
E	63	40·8	2,570	3·52	11·62
F	19	100	1,900	3·58	30·0
G	21	92·5	1,765	2·68	34·5
Combined with other preparations at a similar stage of purification					
H	77	174	13,400	3·28	53·2
K	39	247	9,640	2·80	88·4

* See p. 603.

Stage A. Two fresh ox spleens were stripped of fat and minced (1700 g.). Anhydrous redistilled acetone (3400 ml.)

was stirred into the mince and the mixture was filtered with suction through a soft paper. After repeating the acetone treatment on the filter cake, the latter was broken up and stirred mechanically for 1 hr. with tap water (3400 ml.). After centrifugation the supernatant liquid containing the enzyme was collected and dialyzed overnight against running tap water (A).

Although the precipitation of β-glucuronidase from aqueous spleen extract with acetone led to a loss in activity it was found that minced spleen washed with acetone and then extracted with water yielded about the same quantity of enzyme as the untreated spleen. Such preliminary acetone treatment was found to be advantageous, since it reduced the total protein in the aqueous extract to about one-third.

Stage B. The dialysate (A) was adjusted to pH 5·0 with 4N-acetic acid, when a heavy precipitate formed; 4M-acetate buffer (5 ml./l., pH 5·0) was added and after addition of a few drops of toluene the solution was incubated at 25–30° for 6 hr. (B).

During the incubation period a large amount of dialyzable reducing material was liberated which interfered with the measurement of enzyme activity. Activity and P.N. estimations were carried out on a measured sample of B which had been dialyzed overnight. As is seen from Table 1 removal of the reducing material by dialysis led to a large increase of β-glucuronidase activity; this phenomenon will be discussed later. There was no need to dialyze the main bulk of solution free of reducing material, since later precipitation of the enzyme with ammonium sulphate removed it from the preparation.

Stage C. In order to remove the precipitate of inactive protein which had formed at pH 5·0, 'Standard Super Cel' (25 g./l.), then 'Celite 545' (35 g./l.) (Johns-Manville Corp. filter aids) were stirred into solution B and the mixture was filtered with light suction through a soft paper. The cake was washed with water (200 ml.) and discarded. The combined filtrate and washings, containing the enzyme, was a clear deep red solution (C). A measured sample was dialyzed before carrying out activity and P.N. estimations.

Stage D. Solid ammonium sulphate (215 g./l.) was stirred into solution C. After standing 10 min., 'Standard Super Cel' (10 g./l.), then 'Hyflo Super Cel' (15 g./l.) were stirred in, the mixture was filtered with suction and the cake discarded. The filtrate was made up to 450 g./l. with solid ammonium sulphate and allowed to stand overnight in an ice-chest. Most of the clear supernatant liquid was then siphoned off and the remainder centrifuged. The cake, containing the enzyme, was dissolved in the minimum quantity of water and dialyzed free of ammonium sulphate in running tap water (D).

Little purification was achieved by this step, but a concentrated protein solution was obtained which enabled the later ammonium sulphate fractionation to be carried out efficiently. Removal of inactive protein precipitates has been found to be greatly facilitated by addition of 'Celite' filter aids. Active precipitates, however, were better separated by centrifugation; although they could be filtered off with 'Celite', and could be eluted quantitatively with water, the resulting solutions were more dilute than was desirable for further purification.

In the remaining stages of the procedure advantage was taken of the finding that the variation in solubility of the enzyme at pH's between 4·3 and 7·3 was different from that of much of the inactive protein. Ammonium sulphate fractionation was therefore carried out at three different pH's between these limits.

Stage E. Solution D was adjusted to pH 5·0 with 4N-acetic acid and sufficient SAS (saturated ammonium sulphate solution), previously brought to pH 5·0 with 3N-NaOH, was added to bring the salt concentration to 0·35 SAS. 'Standard Super Cel' (10 g.) was stirred in, the precipitate filtered off at the pump, washed with a few ml. 0·35 SAS and discarded. SAS was added to the combined filtrate and washings at pH 5·0 until it was 0·50 saturated, the resulting enzyme precipitate being centrifuged down, dissolved in water and dialyzed (E).

Stage F. Solution E was made 0·20 saturated with SAS and titrated to pH 4·3 with 4N-acetic acid. A small precipitate was filtered off with suction after addition of 'Standard Super Cel' (1 g.) and discarded. The filtrate was titrated to pH 5·0 with 3N-NaOH and made 0·35 saturated with SAS. The precipitate was filtered off with suction after addition of 'Standard Super Cel' (1 g.), washed with a small amount of 0·35 SAS and discarded. 3N-NaOH was added to the filtrate until the pH was 7·3, when a heavy precipitate formed. The mixture was made 0·36 saturated with SAS, previously titrated to pH 7·3, 'Standard Super Cel' (1 g.) added, and the mixture filtered with suction. The cake was washed with a few ml. 0·36 SAS (pH 7·3) and discarded. SAS was added to the filtrate at pH 7·3 until it was 0·46 SAS, the precipitated enzyme was centrifuged off, dissolved in water and dialyzed free of ammonium sulphate against distilled water (F).

Stage G. After 24 hr. dialysis a flocculent precipitate was centrifuged off and discarded (supernatant solution G).

Stage K. Solution G was combined with several other preparations worked up to the same stage to give solution H. This was adjusted to pH 5·0 with 4N-acetic acid made 0·35 saturated with SAS; a heavy precipitate was filtered off with 'Standard Super Cel' (2 g.) and discarded. The filtrate was made 0·36 SAS at pH 7·3, 'Standard Super Cel' (1 g.) was added and the mixture filtered. SAS was added to the filtrate at pH 7·3 until it was 0·46 saturated, the precipitate containing the enzyme being centrifuged off, dissolved in water and dialyzed against distilled water to give a clear deep brown solution (K).

The value of G.U./mg. P.N. for solution K shown in Table 1 is one of the highest that has yet been obtained. It is seen that the activity/mg. P.N. of this solution is about 125 times greater than that at stage B. If the increase in activity resulting from incubation of the original aqueous spleen extract is taken into consideration the activity/mg. P.N. at stage K is about 315 times that at stage A. Many purified preparations have been made by this method and in general the purification resulting from each step follows closely that shown for the single preparation represented in Table 1. At any stage in the procedure the enzyme appeared to be quite stable for at least a week at 5°. However, 50% of the total activity of solution K (Table 1) was lost by freeze-drying the enzyme. No success has been realized as yet in crystallizing the enzyme. Electrophoretic analysis at pH 7·0 of an enzyme

extract containing 80 G.U./mg. P.N. (kindly carried out by Dr R. A. Kekwick of the Lister Institute) showed that at least two and probably three components with closely similar mobilities at this pH were present.

β-Glucuronidase activity of spleen extracts after incubation at 25° and dialysis

In the work described in the preceding sections it was observed that the β-glucuronidase activity of a spleen extract was increased by 5–7 hr. incubation at 25° followed by dialysis. Fig. 2 represents the results of an experiment designed to illustrate this point.

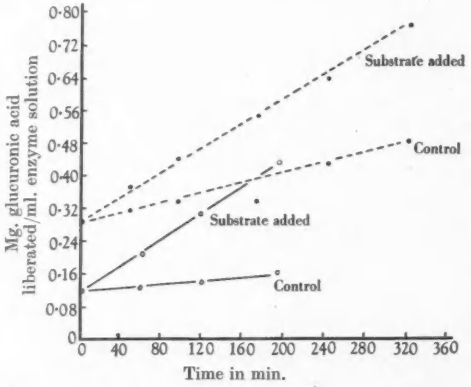

Fig. 2. Progress curves of extract of acetone-treated ox spleen. Broken lines: no preliminary treatment; solid lines: after incubation of extract for 8 hr. and dialysis.

An aqueous extract of spleen (A, Table 1) was divided into two portions. Time-action curves in the presence and absence of substrate at 25° and pH 5·0 were obtained for the first portion and are shown as broken lines in Fig. 2. A similar set of results was obtained with the second portion of extract after it had been incubated at 25° for 8 hr. and dialyzed overnight; this is represented by the solid lines in Fig. 2.

These curves show that the liberation of glucuronic acid from the substrate by incubated and dialyzed enzyme is about 55% more rapid than with the unincubated spleen extract. The first two columns of Table 2 show the activity in G.U./ml. extract for several different enzyme preparations before and after incubation for 6 hr. at 25°, followed by dialysis. Comparison of the figures in these columns shows that there is always a rise in activity after incubation and dialysis of the extract. Columns 3 and 4 indicate that there is an accompanying small decrease in P.N.

Table 2. *Effect of incubation at 25° for 6 hr. and dialysis of spleen extracts on β-glucuronidase activity and protein nitrogen*

	Activity (G.U.*/ml. enzyme solution)		Protein-N (mg./ml. enzyme solution)	
Preparation	Before incubation	After incubation and dialysis	Before incubation	After incubation and dialysis
25	0·130	0·200	2·20	1·54
26	0·190	0·295	2·23	1·54
27	0·060	0·140	—	—
28	0·100	0·150	—	—
29	0·120	0·170	—	—
30	0·095	0·120	—	—
31	0·140	0·180	—	—
33	0·100	0·140	1·43	1·11
34	0·055	0·115	2·16	1·81

* See p. 603.

To determine whether the increased activity of the extract after dialysis was due to the removal of an enzyme inhibitor formed during the previous incubation, in several experiments some of the dialysate was added to the enzyme and time-action curves were determined in the presence of substrate. Comparison of these results with those of a control experiment in which there was no added dialysate showed that there was no inhibition of the enzyme. Moreover, if an inhibitor were being formed during autolysis of the spleen extract, the time-action curve of the extract with added substrate would be expected to deviate from linearity. Inspection of the broken lines in Fig. 2 shows that this in fact does not occur during the period studied. It is hoped that work on the kinetics of the enzyme will provide an explanation for this curious observation.

Specificity of the enzyme

Masamune (1934) claimed that the enzyme prepared by his method was specific for the hydrolysis of β-glucuronides since there was no hydrolysis of menthol α-glucuronic acid, menthol β-glucoside, menthol α-glucoside and methyl α- and β-glucosides. Phenyl β-glucoside was hydrolyzed only to a slight extent. During the course of the present work several different enzyme preparations have been allowed to act on phenyl β-glucoside, salicin and cellobiose at 25° for 2 hr. at pH 5·0 and in no case has any hydrolysis of the glucoside been observed. It thus appears that the purified enzyme contains no β-glucosidase activity and is a specific glucuronidase.

DISCUSSION

Under the conditions of enzyme assay in the present work the final amount of glucuronic acid estimated in the cerimetric titration is seldom more than

100 μg. Since there is a simultaneous liberation of a much larger amount of reducing material by the initial ox-spleen extract independently of substrate hydrolysis, an error of considerable magnitude can occur in the estimation of β-glucuronidase activity. These findings serve to emphasize the inadequacy of an analytical method depending on the estimation of reducing power for determining enzyme activity in tissue extracts. Results obtained from such a non-specific method cannot be entirely free from suspicion unless the assay is properly controlled and the mechanism of the enzyme-catalyzed reaction is known. In the case of β-glucuronidase, unpublished results obtained by Dr G. A. Levvy in this Department show that for a freshly purified enzyme preparation the rate of substrate hydrolysis is the same whether measured colorimetrically by the Tollens reaction or by the cerimetric titration method. Masamune (1934) found that the rate of liberation of menthol was approximately the same as the rate of increase in reducing power and concluded that the chemical change effected in menthol glucuronide by the enzyme was confined to hydrolysis of the glucosidic link. It thus appears that, at least for the last three stages of purification as represented in Table 1, the figures for β-glucuronidase activity do represent the rate of liberation of glucuronic acid from the substrate.

Masamune (1934) reported that the maximum yield of β-glucuronidase from ox kidney was obtained after 72 hr. incubation at 38° of the tissue with twice its weight of water. This was later confirmed by Oshima (1936) for ox spleen and it appears from his figures that this procedure increased the yield of enzyme by at least 100%. The results reported by Oshima to illustrate this increase of activity were obtained on solutions which had been twice precipitated by ethanol after the autolysis period. It is not clear from Masamune's paper whether he introduced a similar stage of enzyme purification between autolysis and activity determination. Fishman (1939*b*), however, was unable to detect this increase in enzyme activity on autolysis of the spleen. In the present work it has been shown that autolysis of an ox-spleen extract for a few hours at 25° increases the β-glucuronidase activity in some cases by as much as 100%. The rise in activity, however, was observed only on dialysis of the autolyzed solution. It is therefore possible that dialysis of the autolyzed solutions in the present work was, in effect, equivalent to the precipitation of the enzyme with alcohol carried out by Oshima at the same stage, in so far as the β-glucuronidase activities of solutions prepared by either technique are higher than those of the original extracts. Fishman's inability to detect this rise was perhaps due to the fact that his assays were carried out directly on the spleen autolysates without preliminary dialysis or purification of the enzyme.

The procedure for purifying the enzyme described above seems to be somewhat more efficient than that of Fishman (1939*a*) in that the loss of total activity is generally about 50% compared to about 85% loss reported by him. Based on the activity of the initial spleen extract the purification effected by the present method is about 315 times, while Fishman reported about 140-fold purification. However, the fact that in the latter work, boiled enzyme controls were used in assaying the initial spleen extracts, and the error introduced by spontaneous liberation of reducing material from the enzyme solution was thus overlooked, suggests that Fishman's figure is incorrect and should be somewhat higher.

SUMMARY

1. A new method for the purification of ox-spleen β-glucuronidase has been devised. The method involves an isoelectric precipitation of inactive protein followed by ammonium sulphate fractionation at pH 4·3, 5·0 and 7·3.

2. The purified enzyme appears to be specific for the hydrolysis of β-glucuronides, since no hydrolysis of three common β-glucosides was observed in the presence of the enzyme.

3. Aqueous extracts of acetone-treated ox spleen when incubated at 25° at pH 5·0 liberate large amounts of dialyzable reducing material, the process being essentially complete after 6–8 hr. incubation.

4. The β-glucuronidase activity of aqueous extracts of acetone-treated ox spleen is increased, in some cases by 100%, by 6–8 hr. incubation at 25° followed by dialysis against water.

Expenses incurred during this work were partially defrayed by grants from the Moray Fund and the Ritchie Fund.

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The Effect of Feeding Cholesterol without Fat on the Plasma-lipids of the Rabbit. The Role of Cholesterol in Fat Metabolism

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(*Received* 26 *April* 1946)

The extensive studies reported on the biological effects of cholesterol administration are chiefly concerned with changes brought about in the lipid composition of tissues. In the blood only the cholesterol fractions have been systematically examined after cholesterol feeding (cf. Anitschkow & Cowdry, 1933); only a few workers have examined the other blood lipids. Page & Bernhardt (1935), who fed rabbits with cholesterol dissolved in olive oil, reported that besides free and esterified cholesterol the phospholipids were increased in the plasma. Weinhouse & Hirsch (1940) found that phospholipids and neutral fats, as well as the cholesterol fractions, were increased in the serum of rabbits after cholesterol feeding. Vermeulen, Dragstedt, Clark, Julian & Allen (1942) also found that there was a great rise in the 'non-cholesterol fraction' of the serum lipids of rabbits fed cholesterol dissolved in sunflower-seed oil. The studies quoted were concerned chiefly with the experimental production of atheroma; the plasma-lipid values were estimated only after a very long period of cholesterol administration and no attempts were made to elucidate the mode of action of cholesterol.

Most of the changes in tissue- and blood-lipids reported as having been brought about by exogenous cholesterol cannot strictly be attributed to cholesterol alone (except those found by Weinhouse & Hirsch), but to cholesterol plus a high fat diet. It was generally held until recently that cholesterol is not appreciably absorbed from the alimentary tract unless fats are administered with it (cf. Bloor, 1943, pp. 106–8). This is apparently true only for crystalline cholesterol, for Dömösi & Egyed (1939) have shown that cholesterol is readily absorbed from the alimentary tract of rabbits if it is administered in the form of an amorphous suspension in water. The total blood-cholesterol level of their rabbits, fed 1 g. of cholesterol daily, rose in 4–5 days from the normal of about 80 to 300–400 mg./100 ml., and in 3–4 weeks the rabbits developed atheroma in the aorta and pulmonary arteries and the total blood-cholesterol levels reached 1300–1800 mg./100 ml. Dömösi & Egyed made no observations on non-cholesterol lipid fractions in the blood and their earliest observations were made after 4 days of cholesterol administration.

The experiments to be reported here were originally designed to study the effect of cholesterol on the kidney lipids. It might be mentioned that while cholesterol readily induces fatty livers—'cholesterol-fatty liver' (Best & Ridout, 1933; Chanutin & Ludewig, 1933; Okey, 1933; Best, Channon & Ridout, 1934; Channon & Wilkinson, 1934)—in the kidney only negligibly small amounts of cholesterol or neutral fat are deposited in the tubular epithelium during cholesterol feeding. In these rabbits' kidneys cholesterol accumulated only in the interstitial cells and capillary endothelium. The plasma-lipids during the experiments were also investigated and results were obtained which are thought to cast some new light on the rather obscure role of cholesterol in fat metabolism.

The changes observed in plasma-lipids in the present experiments can be attributed entirely to the action of cholesterol as no fats, save the small amounts contained in the usual vegetable diet, were administered to the rabbits.

MATERIAL AND METHODS

Rabbits of about 3000 g. weight were used. Their diet consisted of bran, oats, cabbage and water *ad libitum*. They were fed daily through a stomach tube 0·5–1·0 g. of amorphous cholesterol suspended in water. Cholesterol purified by recrystallization was converted into its amorphous form by dissolving suitable quantities in hot glacial acetic acid and pouring the hot solution into a large volume of cold water (cf. Dömösi & Egyed, 1939). Amorphous cholesterol precipitated immediately and the acid was removed by washing several times on a Buchner funnel. Suspensions of the acid-free amorphous cholesterol were prepared in tap water so that 20 ml. of the suspension contained 1 g. of cholesterol.

Blood was taken from the lateral ear-vein at varying intervals. Neutral potassium oxalate (3 mg./ml.) or heparin were used as anticoagulants. The plasma was separated by centrifuging and was immediately extracted.

Analytical procedures. The plasma was extracted by boiling with 30–40 volumes of an ethanol-ether mixture (3 : 1, v/v) by the method of Bloor (1928). The filtered extracts were evaporated to dryness under reduced pressure (20–40 mm. of Hg) at an external temperature of 30–40°. The dry residue was re-extracted with light petroleum (b.p. 40–60°) and the various lipid fractions were isolated from the latter extract. Phospholipids were precipitated with acetone and $MgCl_2$ and were determined by

the dichromate oxidation method of Bloor (1929). The supernatant fluid obtained after phospholipid precipitation was divided into a smaller and a larger sample: the former was used for the determination of free cholesterol and the latter, after saponification, for the determination of total cholesterol and of non-phospholipid fatty acids (derived from neutral fats and cholesteryl esters). For the saponification, the larger sample of the acetone supernatant was first evaporated to dryness on a hot plate and then 5 ml. of ethanol and 0·3 ml. of aqueous KOH, containing 40 g. KOH/100 ml., were added. The mixture was kept in an incubator at 75° for 2 hr. After completion of hydrolysis most of the ethanol was evaporated off on a hot plate and then about 2 ml. of distilled water were added. The last traces of the ethanol were removed by further heating and current of air. The mixture was made acid with 5% (w/v) HCl and extracted with light petroleum. Suitable samples of this extract were used for the determination of total cholesterol and non-phospholipid fatty acids. For the latter, samples of the light petroleum extract were evaporated to dryness on a hot plate and the residue was dissolved in 10 ml. ethanol. The fatty acids were titrated at 75° with 0·02N-aqueous NaOH, using phenolphthalein as indicator. The average molecular weight of the fatty acids was assumed to be 275.

Free and total cholesterol were determined by the digitonin method of Popják (1943).

Ester cholesterol was calculated as the difference between total and free cholesterol, and neutral fat by the formula:

$$\text{neutral fat} = (\text{non-phospholipid fatty acids} - 0{\cdot}72 \times \text{ester cholesterol}) \times 1{\cdot}05.$$

The iodine values were determined by Yasuda's (1931–2) micromodification of the pyridine sulphate dibromide method.

RESULTS

The results obtained were very uniform and for this reason detailed data of representative experiments only will be presented.

Early changes in plasma-lipids after administration of cholesterol

No changes were found in the lipid content of the plasma until 24 hr. after feeding 1 g. of cholesterol, but after 24 hr. the plasma usually appeared slightly opalescent and the analyses showed a considerable increase in all the lipid fractions, e.g. free and esterified cholesterol, phospholipids and neutral fat (Table 1). Even 48 hr. after the single dose of cholesterol the plasma-lipid values were still elevated, but after 1 month without further cholesterol the plasma-lipids of this rabbit returned to slightly below the original levels. There is no explanation available at present of the 24 hr. delay in the plasma-lipid changes after ingestion of cholesterol. Possibly (*a*) in the rabbit the contents of the stomach may be retained for long periods, (*b*) cholesterol, although absorbed earlier than 24 hr. after the administration, may not reach the blood because of storage in the tissues, possibly in the liver. The observation of Aylward, Channon & Wilkinson (1935) that cholesteryl esters in the liver of rats increase steadily from the 4th hr. onwards, after the ingestion of cholesterol-containing food, suggests that probably the second explanation is more valid. This might indicate that the effects of cholesterol on plasma-lipids are mediated through the liver.

Plasma-lipids during prolonged feeding of cholesterol

Two typical experiments are recorded in Tables 2 and 3. It is apparent that after a few days of cholesterol administration high degrees of lipaemia were obtained; the increase in the cholesterol fractions was paralleled by an increase in other lipids. With an increasing lipid content the plasmata became more and more opalescent until finally they resembled milk. Two points in these tables require special mention. When the animals received only 0·5 g. of cholesterol per day their plasma-lipid values were maintained at a high but fairly constant level: at about values recorded in Table 3 after administration of $8 \times 0{\cdot}5$ g. of cholesterol. This indicates that with the daily intake of 0·5 g. of cholesterol there was an equilibrium between absorption and elimination (deposition in tissues, excretion and destruction). The lipaemia, on the other hand, continually increased whenever the

Table 1. *Plasma-lipids of rabbit Ch* 1 *before and after feeding* 1 *g. of cholesterol*

(Values are mg./100 ml. plasma.)

	Non-phospholipid fatty acids	Neutral fat	Phospholipids	Cholesterol		Fatty acids of cholesteryl esters*
				Free	Ester	
Before cholesterol	103	71	102	24	48	35
24 hr. after cholesterol	338	238	207	50	154	111
48 hr. after cholesterol	301	154	224	51	214	154
1 month after cholesterol	84	68	90	16	27	19

* Calculated as ester cholesterol × 0·72.

Table 2. *Plasma-lipids of rabbit Ch* 1 *during prolonged cholesterol feeding* (1 *g. of cholesterol/day*)

(Values are mg./100 ml. plasma.)

Total dose of cholesterol (g.)	Non-phospholipid fatty acids	Neutral fats	Phospho-lipids	Cholesterol		Fatty acids of cholesteryl esters*	Ratio
				Free	Ester		Ester cholesterol/Free cholesterol
None	84	64	90	16	27	19	1·69
2	363	163	227	138	289	208	2·10
6	783	89	592	262	968	698	3·70
8	900	118	600	309	1093	788	3·54

* Calculated as ester cholesterol × 0·72.

Table 3. *Plasma-lipids of rabbit Ch* 5 *during prolonged cholesterol feeding*

(The animal received 0·5 g. of cholesterol per day for the first 8 days; then the dose was increased to 1 g. per day. Values are mg./100 ml.)

Total dose of cholesterol (g.)	Non-phospholipid fatty acids	Neutral fats	Phospholipids	Cholesterol		Fatty acids of cholesteryl esters*	Ratio ester/free
				Free	Ester		
None	181	149	134	22	54	39	2·46
8 × 0·5	395	269	296	132	193	139	1·46
8	975	713	460	214	411	296	1·92
11	955	455	690	366	724	522	1·98
60	1840	368	927	631	1955	1490	3·10

* Calculated as ester cholesterol × 0·72. The concentration of cholesteryl esters can be obtained from the data by the addition of ester cholesterol and fatty acids of cholesteryl esters.

animals were given 1 g. of cholesterol/day; this rise continued for as long as 5–8 weeks, after which a plateau followed by a gradual decline was usually observed. The experiments were not continued beyond 3 months. The plasma-lipid values recorded in Tables 2 and 3 were by no means the highest observed, but an occasional rabbit showed less pronounced increases in plasma-lipids, although no 'resistance' was observed such as that reported by some investigators (cf. Wright, Robinson & Trikojus, 1944).

The second point is that while the phospholipids and non-phospholipid fatty acids (the latter not being a separate lipid fraction, only a convenient laboratory measure) always increased parallel with the rise in cholesterol, the behaviour of neutral fats was variable. In rabbit Ch 1 (cf. Table 2) the neutral fats showed only a moderate to slight increase, while in rabbit Ch 5 (cf. Table 3) there was a far greater increase. It is evident from the last columns of Tables 2 and 3 that the two rabbits in question differed in the degree to which they were able to esterify cholesterol, this being higher in rabbit Ch 1 than in rabbit Ch 5. The same relationship was observed in other animals, and the general conclusion can be drawn that animals which markedly esterify cholesterol will respond with a slight to moderate increase, and those which esterify cholesterol to a lesser degree with a greater rise in plasma-neutral fats during cholesterol administration.

The ratio of ester to free cholesterol commonly increased with the rising of plasma-total cholesterol up to *c.* 1200 mg./100 ml.; when the total cholesterol reached values higher than this, the degree of esterification usually decreased. There were, however, some exceptions to this rule as the example recorded in Table 3 shows.

Mode of action of cholesterol

The fact having been established that the administration of cholesterol causes without exception a great increase in the fatty acid compounds of the plasma, the origin of these fatty acids remains to be explained. Dietary fat can be readily excluded as the source, since the fat content of bran, oats and cabbage is insufficient to account for the increases in the plasma. Two alternatives remain: (*a*) that the fatty acids came from mobilized depot fat, and (*b*) that there was an increased synthesis of fatty acids from carbohydrates. The second alternative could not be investigated owing to lack of facilities. Data will be presented, however, which are thought to show that probably the major portion, if not all, of the fatty acids built into the various lipids of the plasma during cholesterol administration are derived from the fat of connective tissue.

The first indication that connective tissue fat might be the source of the fatty acids in the plasma was provided by direct observation of the fat-depots of the experimental animals. The rabbits used were kept in the department for several months before the experiments, during which time they were fed *ad libitum* and they built up large amounts of adipose tissue. The nutritional state of rabbits can

be well assessed from the total weight of the abdominal and scapular fat (cf. Dible & Popják, 1941). Ten control animals, from the same stock as that used for the cholesterol-feeding experiments, possessed 200–250 g. (fresh weight) of adipose tissue. Twenty-five cholesterol-fed animals, on the other hand, killed at varying intervals during the experiment (3–12 weeks on cholesterol) had only very little (0–50 g.) reserve fat. The depletion of fat depots, moreover, was related to the duration of the experiment: the longer the period of the cholesterol feeding the smaller the amount of the body adipose tissue. The appearance of the remaining fatty tissue was very characteristic: it was slightly oedematous ('gelatinous') and greyish-white or brown, appearances which in the experience of morphologists suggest a rapid depletion.

In spite of the apparent loss of 150–200 g. of adipose tissue the animals either maintained their body weight, or lost only 50–100 g. in the course of several weeks. This may indicate that at the time of increased fat utilization there was a deposition of other body constituents, but it is more probable that the maintenance of body weight (or only slight loss) while the animals lost up to 200 g. of adipose tissue was due to water retention. Determinations of water and electrolyte content of skeletal muscle and serum showed that there was a considerable increase in the volume of the extra-cellular phase of the muscle; the muscles were often visibly oedematous. The changes in water and electrolyte equilibria in these animals will be reported in a later publication.

Although there was no indication that the rabbits consumed less food during cholesterol administration, more exact experiments were made to ascertain that the diminution of the depot fat was not caused by a decreased food intake.

Four rabbits were placed in separate metabolism cages and were given a weighed amount of the stock diet (2 parts oats + 1·5 parts bran) in excess of their requirements; 100 g. (fresh weight) of cabbage were also given daily. The food left over was collected daily and weighed. After a 3-week control period the rabbits were given 1 g. of cholesterol/day and their food consumption and body weight measured.

The results of two typical experiments are shown in Fig. 1, in which the average food consumption and body weights are recorded for 3-day intervals. The recorded food consumption represents the dry weight of the oats and bran mixture only; the rabbits ate all the cabbage offered. The mean food consumption, calculated from 3-day averages, of rabbits 75 and 76 during the control period was 100 ± 6 g. and 102 ± 8 g. respectively, and during cholesterol administration 114 ± 9 g. and 109 ± 14 g. respectively. During the third week of the control period there was a slight fall in food consumption and body weight (Fig. 1) which might have been due to the fact that in the smaller space of the metabolism cages the movements of the rabbits were more limited, resulting in a loss of appetite. After cholesterol feeding was started the daily food intake rose to levels slightly above those of the control period and the body weights were maintained at about the initial levels. After

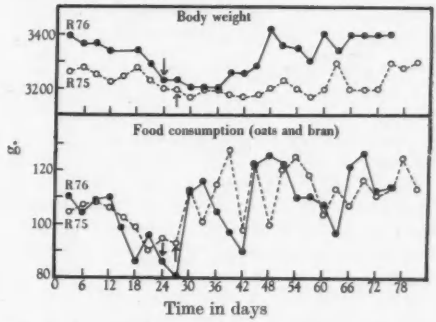


Fig. 1. Food consumption and body weights of rabbits 75 (o - - - o) and 76 (•—•) before and during cholesterol feeding. Cholesterol feeding was started at ↑.

12 weeks on cholesterol the rabbits were killed; the total amount of the dissected adipose tissue (omental, mesenteric, pelvic, perirenal and scapular fat) in rabbit 75 was <10 g. and in rabbit 76, 40 g. The other two experiments yielded essentially the same results. Five of the control rabbits from the same stock, with similar body weights and kept on the same diet, were killed at the same time and all of them had more than 200 g. of fatty tissue.

It is evident therefore that cholesterol-fed rabbits do utilize more fat than those without cholesterol. The amount utilized in excess of normal is however no more than about 1 g./day/kg. rabbit.

Although the evidence brought forward is more circumstantial than direct, the depletion of the fat depots was observed with such regularity that it leaves little doubt in the writer's mind as to its real significance.

Lipids of lymph during cholesterol administration

Observations on the lipid content of lymph during cholesterol administration give further support to the view that cholesterol causes an increased mobilization of depot fat.

The flow of lymph in the cysterna chyli of cholesterol-fed rabbits was so rapid that 1–2 ml. of lymph could be easily collected with a syringe in about 5 min. One representative observation out of three is shown in Table 4, in which the lipid content of lymph and blood plasma obtained simultaneously from rabbit 42 are compared. The striking feature of the comparison is that while the lymph contained less phospholipids and cholesterol, its neutral fat content was nearly five times greater than that of the plasma. Since the animal received no fat in its diet it is inferred that the neutral fat in the lymph

Table 4. *Lipid content of lymph and blood plasma of rabbit* 42, *after receiving* 1 *g. of cholesterol/day for* 1 *month*

(Values are mg./100 ml.)

	Non-phospholipid fatty acids	Neutral fats	Phospholipids	Cholesterol		Fatty acids of cholesteryl esters*
				Free	Ester	
Lymph	770	664	174	102	191	138
Blood plasma	747	140	510	198	852	614

* Calculated as ester cholesterol $\times 0{\cdot}72$.

was derived from the adipose tissues. This observation agrees with the data summarized by Bloor (1943, p. 363) that mobilized fat enters the lymphatics first and is then carried into the blood. That the lipids found in the lymph are not necessarily derived from intestinal absorption was clearly shown with the aid of radioactive phosphorus by Reinhardt, Fishler & Chaikoff (1944), who found that phospholipids are exchanged between blood plasma and lymph. Taking this fact into account, the relatively high phospholipid content of the lymph shown in Table 4 can be accounted for by such an exchange and does not require the postulation of synthesis by the intestinal mucous membrane. The high neutral fat content of the lymph, on the other hand, cannot be explained by the same mechanism, since the concentration in the plasma was less than in the lymph. Whether a circulation of the cholesterol fractions between blood and lymph exists cannot yet be decided.

Source of excess phospholipids during cholesterol administration

It has been shown by Fishler, Entenman, Montgomery & Chaikoff (1943) with the aid of ^{32}P, that plasma-phospholipids are synthesized exclusively by the liver. In the present experiments a ten-fold rise in plasma-phospholipids was not an unusual finding and in view of the work of Fishler *et al.* the cause of this increase must be an increased synthesis of plasma-phospholipids by the liver under the influence of cholesterol. The mean total phospholipid content of the liver of 15 rabbits fed cholesterol for 2–12 weeks was $3{\cdot}39 \pm 0{\cdot}52\%$ and that of 12 normal rabbits $3{\cdot}10 \pm 0{\cdot}25\%$ of the moist weight. The mean weight of the livers of the cholesterol-fed rabbits was 102 ± 24 g. and that of the controls 65 ± 16 g. It may be calculated then that the livers of normal rabbits contained an average of 2·02 g. and those of the cholesterol-fed rabbits 3·46 g. of phospholipid. The increase in plasma-phospholipids, therefore, cannot be regarded as caused by a mere displacement of liver phospholipids by the accumulation of cholesterol in this organ, but must be attributed to new and increased synthesis. These observations may seem contradictory to the findings of Okey (1933, 1944) and Best *et al.* (1934) that the concentration of phospholipids in 'cholesterol fatty livers' is decreased. This discrepancy is probably due to the fact that in these authors' experiments cholesterol was fed in a high fat diet which resulted in a deposition of large amounts of neutral fat as well as cholesterol in the liver. The changes in liver lipids in the present experiments will be reported separately, but it may be mentioned that the feeding of cholesterol alone, without fat, resulted in an accumulation of only small amounts of neutral fat in the liver.

A further discrepancy arises from the finding of Perlman & Chaikoff (1939) that feeding of cholesterol in a high fat diet for 30 hr. depresses the phospholipid 'activity' in the liver of rats, as measured by radioactive phosphorus. They did not measure, however, the radioactivity of plasma-phospholipids and it may well be that under the influence of cholesterol the newly synthesized phospholipids are poured rapidly into the blood.

Iodine values of plasma-fatty acids during cholesterol administration

In an endeavour to obtain further evidence on the source of the plasma-fatty acids, their iodine values were determined in 8 rabbits at weekly intervals during cholesterol administration. It was thought that if the mobilized depot fat fatty acids are built directly into phospholipids the iodine value of the phospholipid fatty acids should approximate that of depot fat fatty acids; if, on the other hand, the cholesteryl esters act as carriers of fatty acids from the depots, their fatty acids should have an iodine value close to that of the fatty acids of adipose tissue.

All the results were the same; two representative experiments are recorded in Tables 5 and 6. During the administration of cholesterol the iodine value of the phospholipid fatty acids decreased markedly, i.e. these phospholipids contained more saturated fatty acids than before the experiment. The changes in the iodine values of these fatty acids recorded in Table 5 were the least observed; in other experiments iodine values as low as 66 were found.

When the administration of cholesterol was discontinued for one week (Table 6), the plasma-lipids

Table 5. *Plasma-lipids and their iodine values in rabbit 53 before and during administration of 1 g. of cholesterol per day*

(The interval between successive determinations was 1 week.)

	Non-phospholipid fatty acids								
	Total		Fatty acids of cholesteryl esters*	Fatty acids of neutral fats†	Neutral fats	Phospho-lipids	Iodine value of phospho-lipid fatty acids‡	Cholesterol	
	(mg./100 ml.)	Iodine value	(mg./100 ml.)	(mg./100 ml.)	(mg./100 ml.)	(mg./100 ml.)		Free (mg./100 ml.)	Ester (mg./100 ml.)
Before cholesterol	98	88·0	25	63	66	134	110·6	20	35
During cholesterol feeding	450	85·0	353	97	102	361	89·5	134	495
	552	98·0	443	109	114	521	84·0	195	615
	1150	99·5	884	266	279	860	87·0	438	1228
	1146	100·6	864	282	296	835	81·9	427	1200
	1261	116·2	1054	207	207	1010	82·0	636	1464

* Ester cholesterol × 0·72.

† Non-phospholipid fatty acids − (ester cholesterol × 0·72).

‡ Calculated from the iodine values of whole phospholipids assuming that fatty acids make up 69% of the phospholipid molecule.

Table 6. *Plasma-lipids and their iodine values in rabbit 75 before and during administration of 1 g. of cholesterol per day*

(The interval between successive determinations was 1 week.)

	Non-phospholipid fatty acids								
	Total		Fatty acids of cholesteryl esters	Fatty acids of neutral fats	Neutral fats	Phospho-lipids	Iodine value of phospho-lipid fatty acids	Cholesterol	
	(mg./100 ml.)	Iodine value	(mg./100 ml.)	(mg./100 ml.)	(mg./100 ml.)	(mg./100 ml.)		Free (mg./100 ml.)	Ester (mg./100 ml.)
Before cholesterol	114	83	46	68	71	113	106	26	64
During cholesterol feeding	686	98	503	183	192	564	75	236	699
	799	100	649	150	157	730	78	312	902
	1048	118	788	260	273	796	72	417	1094
	Cholesterol discontinued for 1 week								
After 1 week without cholesterol	722	118	646	76	79	586	91	281	897
	Cholesterol feeding resumed								
After 1 week of resumed cholesterol feeding	1660	97	1013	647	679	988	72	524	1407

showed a decline and the iodine value of the phospholipid fatty acids increased; on the resumption of the feeding of cholesterol, plasma-lipids rose again and the iodine value of the phospholipid fatty acids decreased. The iodine value of the crude depot fat fatty acids of rabbits fed on bran, oats and beet pulp was found (Dible & Popják, 1941) to be 66 ± 7 (range 60–86). The iodine values of the phospholipid fatty acids during cholesterol administration, therefore, are close to those of the adipose tissue. It is inferred from these findings that the fatty acids of mobilized depot fat have been built into plasma-phospholipids. It is of some interest to note that, while the iodine values of the fatty acids of plasma-phospholipids during cholesterol administration varied between 66 and 80, the iodine value of the fatty acids of liver phospholipids was 95–100. There appears to be a selective 'secretion' into the blood by the liver of the phospholipids containing the more saturated fatty acids.

The iodine values of the non-phospholipid fatty acids showed the reverse of what was found for phospholipid fatty acids. The greater proportion of these fatty acids during the experimental period was derived from cholesteryl esters (cf. Tables 5 and 6) and their iodine values therefore represent more closely those of the fatty acids of cholesteryl esters; whereas before the administration of cholesterol they were more representative of the neutral fat fatty acids. Bloor, Blake & Bullen (1938) have shown for normal human plasma that the degree of unsaturation of the fatty acids increased in the

order: neutral fat < phospholipid < cholesteryl ester. It can be inferred from the data presented that this is also true in the rabbit, except that under the conditions of the present experiments the degree of unsaturation of the phospholipid fatty acids is nearly the same as that of neutral fats, presumably because during the rapid transfer of the fatty acids from neutral fats to phospholipids no significant degree of unsaturation occurred.

If the fatty acids of cholesteryl esters were ultimately also derived from adipose tissue fat, as the depletion of fat depots suggests, a considerable degree of unsaturation had taken place. Whether this unsaturation is a preliminary step in the breakdown of the fatty acids cannot be decided. The conflicting views on the subject have been recently reviewed by Bloor (1943, pp. 314–17).

relationship between blood-total cholesterol and phospholipids was found by Mayer & Schaeffer (1913) and Bloor (1914), and between total cholesterol and total fatty acids by Terroine (1914), Bloor (1914) and Bang (1918), and more recently by Peters & Man (1943). These relationships are relatively constant among members of the same species in the postabsorptive state.

The data of the experiments were analyzed in respect of these ratios (total cholesterol/phospholipid; total cholesterol/total fatty acids), but it was found that, while the ratios were fairly constant for normal animals, in cholesterol-induced lipaemia no such constancy existed. When plasma-total cholesterol values were plotted against phospholipids, or against non-phospholipid fatty acids (or total fatty acids), the points approximated to a smooth

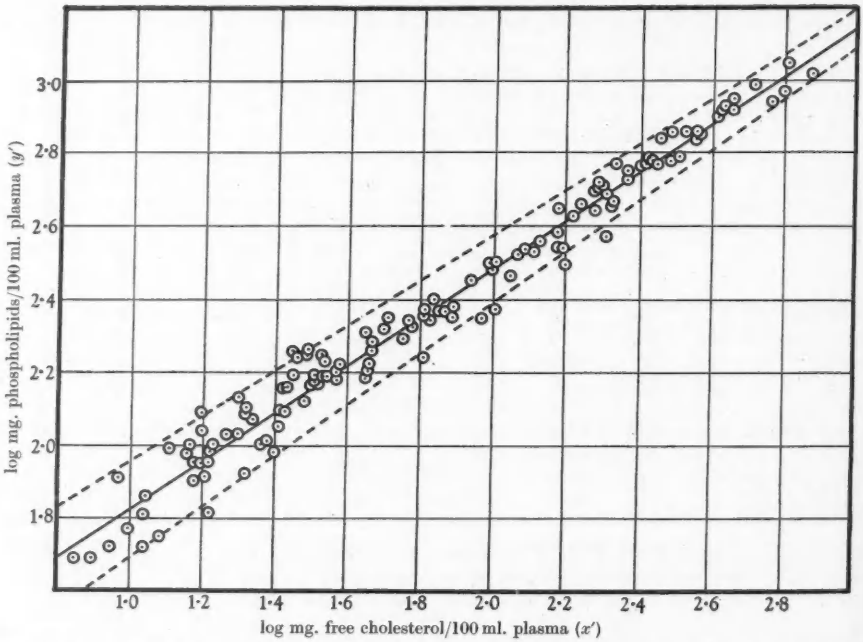


Fig. 2. Relationship between log plasma free cholesterol (x') and log phospholipids (y') in normal and cholesterol-fed rabbits. The continuous straight line represents the equation $y' = 0{\cdot}660x' + 1{\cdot}1536$ calculated by the method of least squares. The interrupted lines, showing the maximum limits of variation, represent the equation

$$y' = (0{\cdot}660 \mp 0{\cdot}03)\, x' + (1{\cdot}1536 \pm 0{\cdot}15).$$

Interrelation between cholesterol and plasma-lipids. Universality of the relationship

It was thought that if cholesterol is the cause of the described changes in plasma-lipids, a quantitative relationship should exist between plasma-cholesterol and the other lipids. A fairly constant

curve (not a straight line) up to total cholesterol values of 600–800 mg./100 ml. Above these values, however, no definite relationships could be detected. It is of some interest, therefore, that a quantitative relationship was found between plasma free cholesterol and phospholipids, and free cholesterol and non-phospholipid fatty acids, over the entire

range of the experimental values. Moreover, as will be shown, the equations expressing these relationships are applicable not only to the data of this investigation, but hold true for values of a number of species in the postabsorptive state and even in some pathological conditions.

A linear relationship was found between the common logarithm of plasma free cholesterol and the logarithm of phospholipids on the one hand, and the logarithm of non-phospholipid fatty acids on the other. The first relationship is illustrated in Fig. 2,

The similar equation relating the logarithms of plasma free cholesterol and of non-phospholipid fatty acids (Fig. 3), calculated also by the method of least squares, was found to be

$$z' = 0{\cdot}752x' + 1{\cdot}061, \qquad (3)$$

where z' = log non-phospholipid fatty acids (mg./100 ml.). The maximum variations from the mean are given by the equation

$$z' = (0{\cdot}752 \mp 0{\cdot}046)\, x' + (1{\cdot}061 \pm 0{\cdot}217). \qquad (4)$$

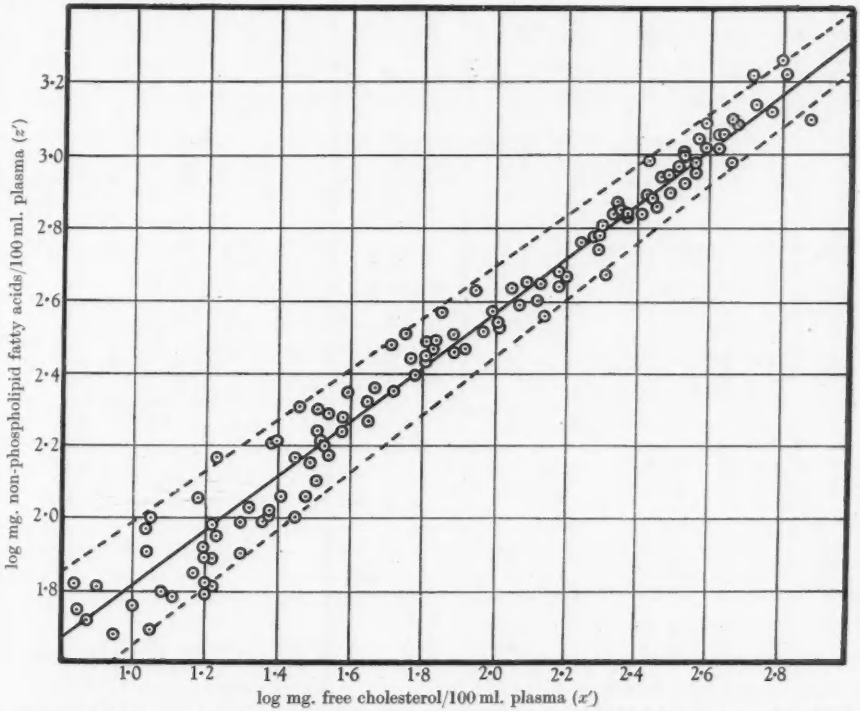


Fig. 3. Relationship between log plasma free cholesterol (x') and log non-phospholipid fatty acids (z') in normal and cholesterol-fed rabbits. The continuous straight line represents the equation $z' = 0{\cdot}752x' + 1{\cdot}061$ calculated by the method of least squares. The interrupted lines, showing the maximum limits of variation, represent the equation $z' = (0{\cdot}752 \mp 0{\cdot}046)\, x' + (1{\cdot}061 \pm 0{\cdot}217)$.

in which the continuous straight line represents the equation calculated by the method of least squares

$$y' = 0{\cdot}660x' + 1{\cdot}1536, \qquad (1)$$

where y' and x' are the common logarithms of plasma-phospholipid and plasma free cholesterol (y and x, both as mg./100 ml.) respectively. The two interrupted lines, giving the maximum observed variations from the mean, correspond to the equation

$$y' = (0{\cdot}660 \mp 0{\cdot}03)\, x' + (1{\cdot}1536 \pm 0{\cdot}15). \qquad (2)$$

By taking the antilog of equations (1) and (3) equations of the type $y = ax^b$ are obtained, which express the concentrations of plasma-phospholipids (y) and of non-phospholipid fatty acids (z) as functions of plasma free cholesterol (x):

$$y = 14{\cdot}240x^{0{\cdot}660}, \qquad (5)$$

$$z = 11{\cdot}508x^{0{\cdot}752}. \qquad (6)$$

These equations together with the observed points are illustrated in Figs. 4 and 5, where it can be seen that about 80 % of all the points fall within ± 10 %,

and 90% within ±15% of the values given by equations (5) and (6). In a preliminary communication (Popják, 1945) slightly different numerical values were given for the coefficient and exponent of x in equation (5). Since then more data have been obtained which allowed the more accurate calculation of these constants.

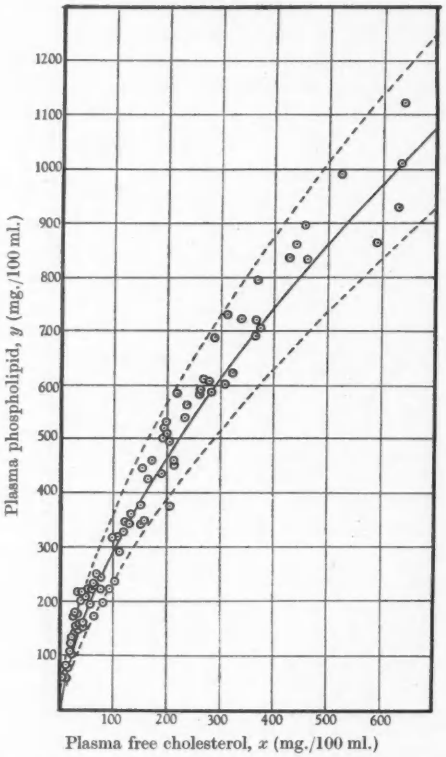


Fig. 4. Relationship between plasma free cholesterol and phospholipids in normal and cholesterol-fed rabbits. The continuous curve represents the equation $y = 14{\cdot}24x^{0{\cdot}666}$.

It follows from equations (5) and (6) that a similar quantitative relationship must exist between plasma free cholesterol and plasma-total fatty acids (Z) also, which should be represented by the equation

$$Z = 20{\cdot}797x^{0{\cdot}720},$$

as derived from (5) and (6).

These results would be of a very limited interest if they were applicable only to rabbits under the conditions of the present experiments. Plasma-lipid values for a variety of species reported by other workers were collected and compared with values defined by equations (5) and (6). These data are shown in Table 7 and illustrated in Figs. 6 and 7. It should be mentioned that the different methods employed for lipid determinations by various

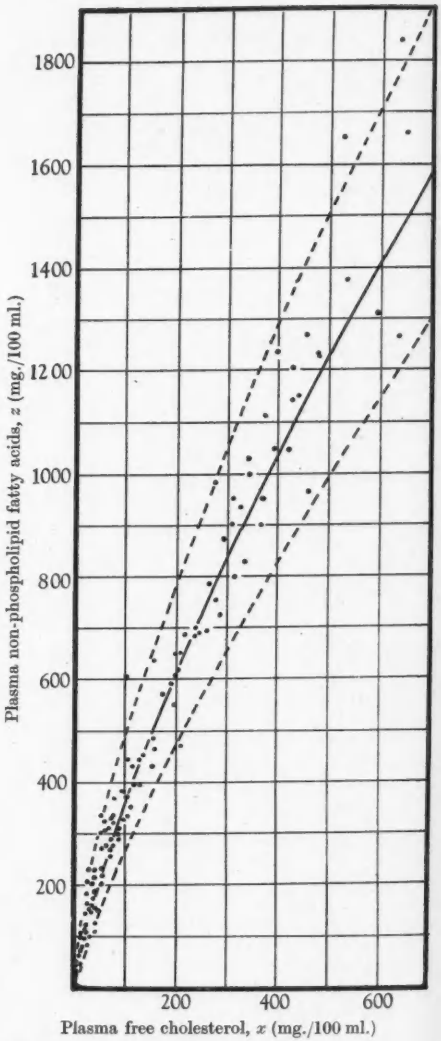


Fig. 5. Relationship between plasma free cholesterol and non-phospholipid fatty acids in normal and cholesterol-fed rabbits. The continuous curve represents the equation $z = 11{\cdot}508\ x^{0{\cdot}752}$.

investigators may give different results (cf. Bloor, 1943, pp. 121–22); for this reason, only those data were selected from the literature which were obtained on plasma by methods similar to those used

Table 7. *Mean plasma free cholesterol, phospholipid and non-phospholipid fatty acid values in different species as found by various investigators*

(Values are mg./100 ml.)

Species	Condition	Free cholesterol	Phospholipids	Non-phospholipid fatty acids	Author
Human adult	Normal	47±7	196±23	228*	Boyd (1936)
Human newborn	Normal	14±7	61±32	98*	
Human adult	Normal	53±10	195±37	227*	Boyd & Connell (1937)
Human adult	Hyperthyroidism	41±8	125±35	178*	
Human adult	Normal	50±12	190±34	—	Boyd (1937)†
Human	Nephritis	74	233	—	
Human	At term of pregnancy	65±15	248±43	—	Boyd (1934)
Human	Normal	70·2±13·9	235±40·1	—	Davis, Stern & Lesnick (1937)
Human	Arteriosclerosis and angina pectoris	86·4±14·1	280±38	—	
Human	Normal	46±8	165±28	202*	Boyd (1942)
Cockerel	Normal	34±9	155±34	254*	
Cat	Normal	30±10	132±53	137*	
Albino rat	Normal	21±8	83±24	95*	
Guinea-pig	Normal	11±2	51±12	81*	
Sheep	Normal and in pregnancy	16±9	90±33	67±20	Barcroft & Popják (1944–5)
Rabbit	Normal	22±9	118±46	117±66	From data of this investigation
Rabbit	On 28th day of pregnancy	5±1	20±5	43±5	Popják (1946)

* These values were calculated when possible from the author's data by the formulae: (neutral fat ×0·95 + ester cholesterol ×0·72), or (total fatty acids − phospholipids ×0·69).

† These data were presented in charts; these were magnified and values read off the scale.

in this investigation. The continuous curves in Figs. 6 and 7 are the initial portions of equations (5) and (6) drawn on a large scale. Within the range represented fall all the normal and some pathological values for the various species. It can be seen that there is very good agreement between the curves obtained from this investigation and the values reported by other workers for a variety of species (human, cat, cockerel, rat, guinea-pig, rabbit, and sheep). It remains to be seen how far these equations can be applied to pathological conditions, but, from a limited number of publications which give the results of differential lipid analyses by methods similar to those employed by the author, it appears that equation (5) expresses the relationship between plasma free cholesterol and phospholipids even in some pathological conditions. In Fig. 8 cases of diabetes mellitus, subacute nephritis and myxoedema are illustrated. The values reported by Rubin (1939) are of particular interest as they were obtained on one person, a 14-year-old diabetic girl, during her recovery from diabetic coma. The phospholipid values were calculated by Rubin from lipid phosphorus, a procedure which yields usually 10–15 % higher values than Bloor's dichromate oxidation method. Taking this into account, there is an excellent agreement with the experimental data.

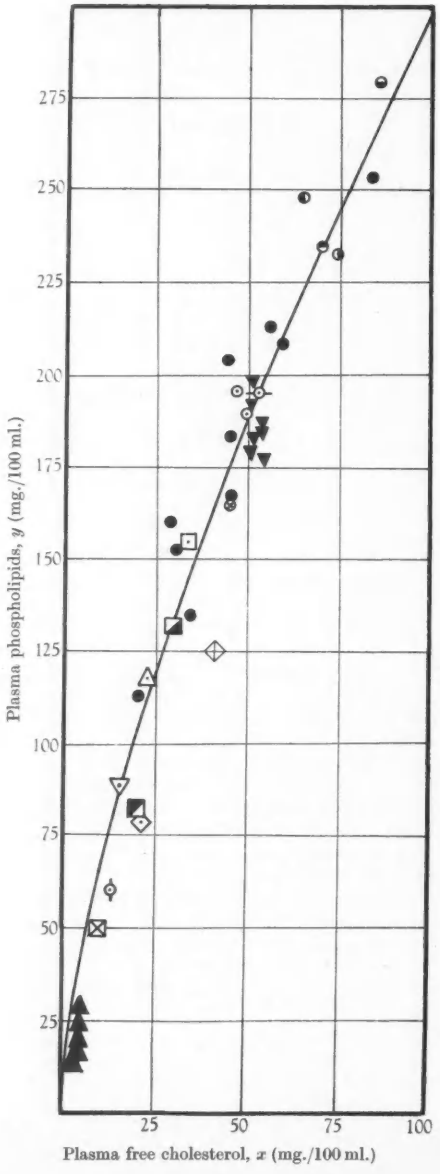
Equation (6) expresses the relationship between plasma free cholesterol and non-phospholipid fatty acids in the postabsorptive state and in such metabolic disturbances as hypo- and hyperthyroidism, but the equation cannot be applied to conditions in which a disturbance of the blood neutral fats occurs, e.g. diabetes mellitus.

It is realized that the numerical values of the exponent and coefficient of x in equations (5) and (6) depend on the methods employed for the determination of the lipids. This point may be illustrated with some further data from the literature. In fig. 9 the log of serum free cholesterol (x') is plotted against the log of serum lipoid phosphorus (p') as calculated from the values found by Man, Kartin, Durlacher & Peters (1945) in patients with various forms of liver disease. It can be seen that there is a good approximation to linearity although there is a greater scattering of the points than in Fig. 2, in which the results of the cholesterol-feeding experiments were illustrated. At least part of this scattering is probably due to a subjective error on the writer's part since the values were presented by Man *et al.* in small-scale charts from which it was

difficult to read off the figures accurately. The straight line in Fig. 9 was drawn visually as the best fit to the points and its equation

$$p' = 0{\cdot}819x' - 0{\cdot}528 \qquad (7)$$

was obtained graphically.



It follows that the antilog of equation (7) should express serum lipid phosphorus (p) as function of serum free cholesterol (x):

$$p = 0\ 297x^{0{\cdot}819}. \qquad (8)$$

DISCUSSION AND CONCLUSIONS

The results presented confirm the finding of Dömösi & Egyed (1939) that fat is not required for the absorption of cholesterol. The concentration of plasma cholesterol reaches much higher values and in a considerably shorter time after the administration of amorphous cholesterol than after feeding cholesterol dissolved in fat. Moreover by eliminating the necessity of a high fat diet the method permits the study of the effects of cholesterol alone.

It is concluded from the results obtained that cholesterol initiates a series of events which manifest themselves in an increased mobilization of depot fat, an increased synthesis of plasma-phospholipids by the liver and an increased utilization of fatty acids. The low iodine values of the phospholipid fatty acids during cholesterol administration, coinciding with the depletion of depot fat, are taken to indicate that these phospholipids were synthesized from the fatty acids of adipose tissue. The high iodine value of the fatty acids of cholesteryl esters suggests that it is unlikely that these esters act merely as carriers of fatty acids from the depots. If desaturation is the primary step in the breakdown of fatty acids, it appears more probable that cholesteryl esters are intimately linked with fatty acid oxidation (cf. Bloor, 1943).

The effects of exogenous cholesterol are considered to shed light on a general function of cholesterol in the normal organism, since it was found that plasma-phospholipid and non-phospholipid fatty acid levels can be expressed as functions of plasma free cholesterol, not only in cholesterol-fed animals, but also in a variety of species in the postabsorptive state.

Fig. 6. Relationship between plasma free cholesterol and phospholipids in different species in the postabsorptive state and in some pathological conditions. The continuous curve represents the equation $y = 14{\cdot}24x^{0{\cdot}660}$ obtained from the cholesterol-feeding experiments. The points are those reported by various investigators. Key to symbols: ⊙ human adult (Boyd, 1936, 1937); ⊙ human new-born (Boyd, 1936); ▼ human, diurnal variations (Boyd, 1935); ⊙- human adult, normal and ⊕ in hyperthyroidism (Boyd & Connell, 1937); ◓ human adult, normal and ◒ in arteriosclerosis and angina pectoris (Davis, Stern & Lesnick, 1937); ◐ human in pregnancy (Boyd, 1934); ◑ human in nephritis (Boyd, 1937); ⊗ human, ⊡ cockerel, ◪ cat, ◩ albino rat, ⊠ guinea-pig, ◇ rabbit (Boyd, 1942); △ rabbit (this investigation); ▲ rabbit on 28th day of pregnancy (Popják, 1946); ● human, individual values (own data); ▽ sheep (Barcroft & Popják, 1944–5).

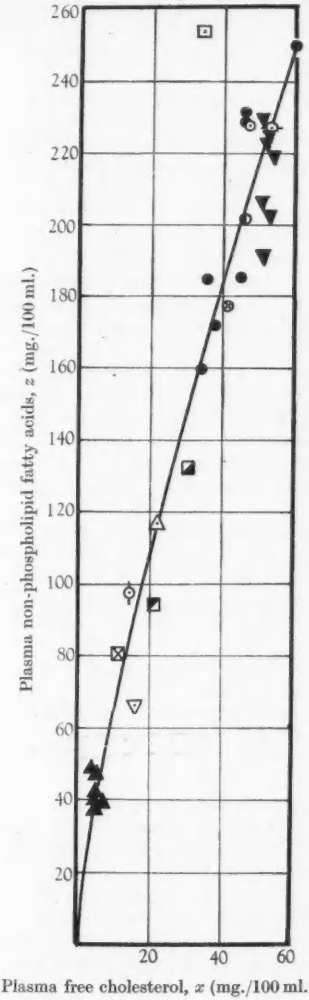


Fig. 7. Relationship between plasma free cholesterol and non-phospholipid fatty acids in various species. The continuous curve represents the equation $z = 11{\cdot}508x^{0{\cdot}752}$ obtained from the cholesterol-feeding experiments. The values shown are those reported by different investigators, or were calculated from their data. Key to symbols: ⊙ human adult and ⊙ new-born (Boyd, 1936); ⊙ human adult normal and ⊗ in hyperthyroidism (Boyd & Connell, 1937); ◐ human, ⊡ cockerel, ◪ cat, ◩ albino rat, ⊠ guinea-pig (Boyd, 1942); ▽ sheep (Barcroft & Popják, 1944–5); △ rabbit (this investigation); ▲ rabbit on 28th day of pregnancy (Popják, 1946); ● human, individual values (own data); ▼ human, diurnal variations (Boyd, 1935).

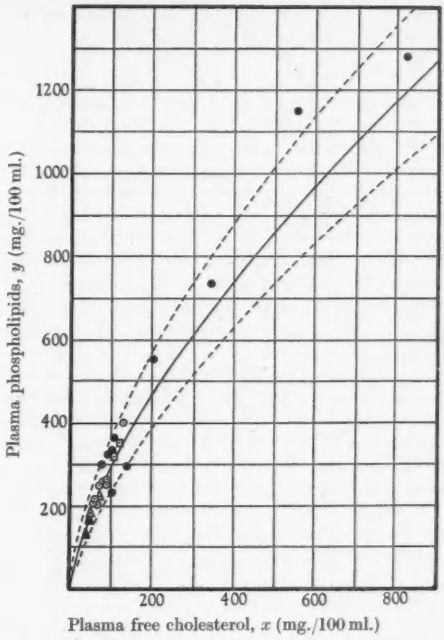


Fig. 8. Relationship between plasma free cholesterol and phospholipids in cases of diabetes mellitus, subacute nephritis, myxoedema, and thyrotoxicosis. The curves are the same as in Fig. 4. Key to symbols: ● diabetes mellitus during recovery from coma (Rubin, 1939); ⊡ subacute nephritis, △ treated diabetes mellitus, ⊙ myxoedema, ▲ thyrotoxicosis (own data).

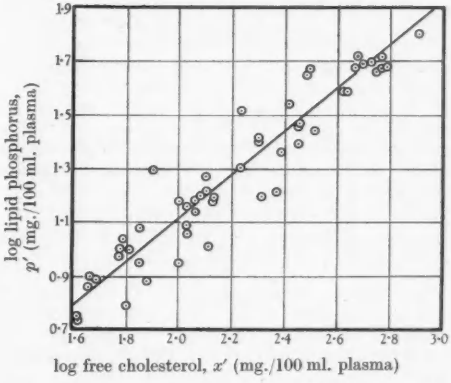
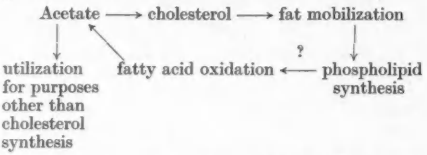


Fig. 9. Relationship between log mg./100 ml. serum free cholesterol (x') and log mg./100 ml. serum lipid phosphorus (p') in cases of liver diseases in human patients. Log values calculated from data of Man *et al.* (1945). The straight line was drawn visually as the best fit to the points and is given by equation $p' = 0{\cdot}819x' - 0{\cdot}528$.

Bloor (1914) was led to the belief, from the analysis of the lipids of a large number of human blood samples, that in the postabsorptive state there is a very efficient regulation of the lipid constituents of the blood. It is suggested here that the regulator of the plasma lipid levels is cholesterol, on the basis of its actions observed in the present experiments, and that this biological principle is common to many species. The fact that the quantitative relationship between plasma free cholesterol and phospholipids is the same even in some pathological conditions as in the cholesterol-feeding experiments and in the normal post-absorptive state, suggests that in these pathological conditions the primary disturbance is an increased synthesis of cholesterol.

If the present findings are correlated with the recent discovery by Bloch & Rittenberg (1942, 1944, 1945) that cholesterol is synthesized from acetate in the animal body, the different normal plasma-lipid levels of the various species (and in some pathological conditions) could be explained by a single factor, i.e. by the different amounts of acetate available for cholesterol synthesis. This hypothesis can be represented schematically:



Experiments which will be reported later, show that this hypothesis in essentials is correct, since hypercholesterolaemia has been produced in experimental animals by the feeding of sodium acetate. The way in which this controlling mechanism is linked with other factors influencing fat metabolism (lipotropic factors, etc.) cannot be conjectured.

SUMMARY

1. The effects of feeding cholesterol, in the absence of a high fat diet, on the plasma-lipids of rabbits were studied.

2. Amorphous cholesterol fed in a watery suspension is well absorbed by rabbits and causes a rise in the free and esterified cholesterol, phospholipids and neutral fats of the plasma 24 hr. after the ingestion. During prolonged cholesterol feeding all plasma-lipids show a progressive increase.

3. The advantages of feeding amorphous cholesterol without added fat are (*a*) that the effects of cholesterol alone can be studied, and (*b*) that induced lipaemia develops more rapidly and reaches higher levels than with the customary high fat cholesterol diets.

4. The fat reserves of the cholesterol-fed animals became depleted in 3–12 weeks in spite of an undiminished food intake.

5. The concentration of neutral fat was higher in the lymph than in the plasma of cholesterol-fed rabbits, indicating an increased mobilization of depot fat.

6. The iodine values of the phospholipid fatty acids of the plasma decreased during cholesterol feeding, nearly reaching those of the rabbits' adipose tissue; on the other hand, the iodine values of the non-phospholipid fatty acids increased. It is suggested that the fatty acids of plasma-lipids during cholesterol administration were derived from mobilized depot fat.

7. Quantitative relationships were found between plasma free cholesterol (x) and phospholipids (y) on the one hand, and non-phospholipid fatty acids (z) on the other. These relationships, in the rabbit, can be expressed by the equations $y = 14{\cdot}24x^{0{\cdot}660}$ and $z = 11{\cdot}508x^{0{\cdot}752}$ respectively. It is shown with the aid of data reported by others that these equations are applicable also to plasma-lipid values of species other than rabbit, including human, in the post-absorptive state and also in some pathological conditions.

8. It is inferred that plasma free cholesterol acts as a regulator of plasma-lipid levels by determining the rate of mobilization of fat from the depots, and the synthesis of plasma-phospholipids. Cholesteryl esters may play a part in fatty acid oxidation.

9. A hypothesis is advanced which seeks to explain the different plasma-lipid levels in the various species by a single factor, i.e. by the different amounts of acetate available for cholesterol synthesis.

I wish to thank Miss Muriel Kendall for her skilful technical assistance.

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The Mechanism of Selective Fermentation of *d*-Fructose from Invert Sugar by Sauternes Yeast

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(*Received* 15 *May* 1946)

It has long been known that brewer's and baker's yeasts preferentially ferment glucose from a mixture of equal parts of *d*-glucose and *d*-fructose, whereas Sauternes yeast selects *d*-fructose (for literature cf. Sobotka & Reiner, 1930*a*). As was shown previously (Gottschalk, 1943*a*), of the various *d*-fructose modifications present in a fructose solution at equilibrium, only fructofuranose is a suitable substrate for fermentation. This reactive substance, constituting approximately 22% of the total fructose concentration at 25° (Gottschalk, 1943*b*), has about twice the affinity of *d*-glucose for hexokinase (Gottschalk, 1944). These findings satisfactorily explain the selective fermentation, at room temperature, of glucose from invert sugar by brewer's and baker's yeasts as the result of the unequal distribution of hexokinase between its substrates *d*-glucose and *d*-fructofuranose, the higher concentration of the aldehyde sugar outweighing the greater affinity of fructofuranose for hexokinase (Gottschalk, 1945*a*). In conformity with this interpretation it was found possible to equalize the relative rates of fermentation by baker's yeast of *d*-glucose and of *d*-fructose from invert sugar by raising the proportion of fructofuranose without changing the total concentration of *d*-fructose (Gottschalk, 1945*b*).

The object of this communication is to investigate the factor or factors responsible for the phenomenon that Sauternes yeast in contrast to the industrial yeasts preferentially ferments *d*-fructose from invert sugar.

Materials and Methods

A pure culture of Sauternes yeast was obtained from the collection of the Institut Pasteur, Paris, through the courtesy of Professor R. H. Hopkins, Birmingham. A stock culture of champagne yeast (*Saccharomyces ellipsoideus*) was kindly supplied by the Roseworthy Agricultural College, South Australia. Both yeast strains were kept in an active condition by subculturing on grape juice-agar slopes (1·5% agar; pH 5·8). For the experiments cultures were grown at 27° in grape juice cleared and sterilized by repeated filtration through Seitz pads. After three 48 hr. passages of the culture through 10 ml., 10 ml. and 100 ml. of the medium respectively, the yeast was sown into a series of 'medicine flats' (450 ml. volume) each containing 100 ml. of medium. After incubation for 4 days the contents of the bottles were pooled, the yeast centrifuged off at 5°, washed twice with water on the centrifuge, once on the Buchner funnel and then compressed. For experiments with living yeast the cake thus obtained was stored at 4° and used within the next three days. Brewer's (ale) top yeast (*Sacch. cerevisiae*) was collected at the Carlton Brewery, Melbourne, from the vats 64 hr. after pitching and was washed and compressed as above. The dry weight of the compressed yeast is recorded in Table 3. Both the wine and

the brewery yeasts showed a very low count (1–2%) of stainable cells after standard treatment with methylene blue.

Dried yeast was prepared in the following manner: The yeast cake, the thickness of which did not exceed 4 mm., was broken up into small fragments and immediately freeze-dried in a desiccator (conc. H_2SO_4 and soda-lime) evacuated to 0·005 mm. Hg. The evacuated desiccator was kept for 19 hr. at 20°, then opened and the dried yeast particles were thoroughly ground for 10–15 min. in a mortar. The resulting extremely fine powder contained less than 0·3% water; upon treatment with methylene blue, 1% of the cells was found to be unstainable. When stored in an evacuated desiccator at 2° this preparation retained its fermenting qualities nearly unchanged for several days.

The substrates used were pure, anhydrous substances:

$[\alpha]_D^{20°}$ glucose: +52·4° (water; $c = 4·05$, $l = 2$).

$[\alpha]_D^{21°}$ fructose: −91·2° (water; $c = 3·09$, $l = 2$).

The concentrations of fructofuranose given in this paper are calculated on the basis that a *d*-fructose solution at equilibrium at 25° contains 22% of the sugar in the furanose form (cf. above).

In the experiments with living cells 1·3–2 g. (unless otherwise stated) of the fresh yeast were suspended in 0·05 M-KH_2PO_4; total volume = 100 ml. 20·0 ml. of this suspension (pH = 4·5) were used in each experiment. The rate of fermentation at 25·0° ± 0·1 was determined with the apparatus described previously (Gottschalk & Rawlinson, 1942). After equilibration of the suspension at 25° with nitrogen (freed from oxygen) as the gas phase, the sugar, dissolved in 1·2–1·6 ml. H_2O and allowed to mutarotate for 24 hr. at 25°, was tipped into the yeast suspension. Readings were taken at 5 min. intervals over a period of 50 min. and the values observed reduced to N.T.P.

With dried yeast as fermenting agent and with glucose and with fructose as separate substrates 0·8–1·2 g. of the yeast preparation was added in an atmosphere of CO_2 to a solution of the hexose in water, prepared 24 hr. previously and saturated with CO_2. After the addition of 10 mg. hexosediphosphate and 0·3 ml. toluene the suspension was placed in the bulb of the respirometer and the gas-space filled with CO_2. After equilibration 0·40–2·20 ml. 0·55 M-K_2HPO_4, saturated with CO_2, and 0·30 ml. 1% (w/v) acetaldehyde were projected into the suspension and readings made at 2 min. intervals. Volume of the liquid phase of the suspension = 10·0 ml.; pH of the suspension at the beginning of the experiment varied from 6·18 to 6·48 with the concentration of orthophosphate added, and the corresponding pH values after 22 min. fermentation ranged from 5·80 to 6·02. Under the conditions described the maximum rates of fermentation were usually attained within the first 6 to 8 min. of the experiment. No correction was applied for the amount of CO_2 released from the bicarbonate of the system due to the formation of hexosephosphoric acids, because the actual pH values of the suspension at the time of the readings, necessary for the calculation of this correction, were not known. Since, however, in the fermentation by dried yeast of *d*-glucose and of *d*-fructose with equal initial concentrations of orthophosphate, no significant difference in the pH levels between glucose and fructose was found after the same period of fermentation, the uncorrected maximum fermentation rates attained under these conditions are comparable. With different initial orthophosphate concentrations different amounts of CO_2 are released from bicarbonate during fermentation. From a rough calculation it appears that these variations in the amount of extra CO_2 liberated are not large enough to prevent a qualitative comparison of the uncorrected maximum fermentation rates with different initial phosphate concentrations.

When invert sugar was fermented by dried yeast, conditions differed from those described above in that 1·2–1·5 g. of the yeast preparation were used and no hexosediphosphate added; the volume of the liquid phase of the suspension used = 15·0 ml.; initial orthophosphate concentration = 0·04 M. After the production of 85–95 ml. CO_2 the fermentation was interrupted by freezing the yeast suspension siphoned into a centrifuge cup. The frozen mixture was then centrifuged as previously described (Gottschalk, 1941) and the supernatant liquid (A) stored at 0° until further treatment. This experiment was carried out in duplicate and the liquids A united. The total amount of the residual glucose plus fructose in A was determined by fermentation, using fresh baker's yeast and ascertaining in a separate experiment that under the conditions prevailing added glucose or fructose is converted 100% into CO_2 and alcohol. Fermentation was carried out with 8·0 ml. of A after adding 120 mg. KH_2PO_4 and 0·20 ml. 1 N-acetic acid (pH = 5·1). The optical rotation of the liquid A before and after removal of the residual hexose mixture was then determined with a Hilger Barfit Polarimeter (source of light: sodium lamp). For this purpose glycogen and any other polysaccharides were precipitated by alcohol (final concentration 55% v/v), the alcoholic solution was concentrated to one-fifth of its volume in the vacuum of a mercury pump at a temperature not exceeding 25° and the proteins left were removed by trichloroacetic acid (final concentration 1·8% w/v). As was seen from control experiments trichloroacetic acid in less than 2% (w/v) concentration does not affect appreciably the specific rotation of *d*-glucose and *d*-fructose. In experiments with living yeast fermenting invert sugar incompletely, the liquid containing the fructose and glucose left was analyzed in a similar manner.

Dried Sauternes yeast—to a much less extent dried Carlton ale top yeast—releases into the suspension fluid a non-reducing, highly dextrorotatory substance which on the evidence obtained (cf. next section) is most probably trehalose. One small fraction of this substance is split into glucose by the yeast preparation during the fermentation of invert sugar, another one is fermented to CO_2 and alcohol by the baker's yeast. To allow for this, a blank consisting of dried Sauternes yeast suspended in water was run with each experiment and treated as above. It was found that 15–22 mg. glucose were produced from this source by dried Sauternes yeast and that fresh baker's yeast ferments within the experimental time an amount of this optically active substance equivalent to a decrease in optical rotation by 0·30°/dm. The results obtained in the main experiment were corrected accordingly. In the case of our ale yeast the corrections were minute.

Orthophosphate determinations were carried out by the method of Kuttner & Cohen (1927). 1 g. pressed yeast and 280 mg. dried yeast respectively were suspended in water (total volume 12·0 ml.) and incubated for 20 and 10 min. respectively at 25°. After adding 3·0 ml. 25% (w/v) trichloroacetic acid, mixing and centrifuging, an aliquot portion of the supernatant was analyzed for orthophosphate.

All pH measurements were made with the glass electrode.

RESULTS

Table 1 shows the effect of substrate concentration on the fermentation rate by living brewer's top, Sauternes and Champagne yeasts. Taking as standard the fermentation rate with 0·049 M-fructofuranose, Sauternes yeast ferments 0·028 M-glucose

Table 1. *Relative rates of fermentation by living yeasts (brewer's, Sauternes, and Champagne) of* d*-glucose and of* d*-fructose with varying substrate concentration (in nitrogen at 25°)*

Type of yeast	*d*-Glucose				*d*-Fructofuranose	
	0·028 M	0·056 M	0·252 M	0·446 M	0·019 M	0·049 M
Brewer's top yeast	81	89	98	98	90	100
Sauternes	59	59	64	66	100	100
Champagne	—	63	78	83	92	100
Champagne (adapted)	—	87	100	—	95	100

at half and 0·446 M-glucose at $\frac{2}{3}$ of this rate, whereas brewer's yeast ferments glucose under the same conditions at $\frac{4}{5}$ and $\frac{5}{5}$ respectively of the standard (cf. Hopkins, 1931). The rates of glucose fermentation by Champagne yeast are intermediate between those of Sauternes and brewer's yeast. The last experiment in Table 1 refers to a strain of the same Champagne yeast that had been passed through 90 subcultures in a broth containing glucose as the only fermentable sugar.

Figs. 1 and 2 demonstrate the variation in the maximum fermentation rate of *d*-fructose and of *d*-glucose by dried brewer's yeast with the concentration of orthophosphate added. As is evident from the curves, the maximum fermentation rates both with fructose and with glucose as substrate increase with rising phosphate concentration to approach a limit. This limit is higher with fructose

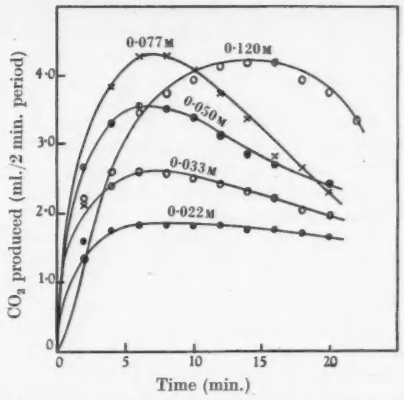


Fig. 1. Rates of fermentation of fructose by dried brewer's yeast plotted against time. Orthophosphate concentration as marked on curves.

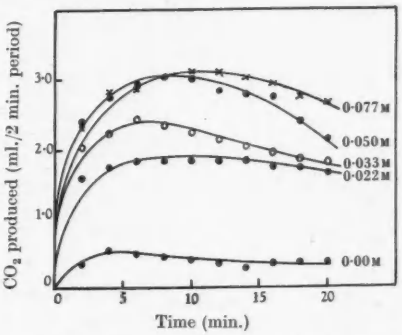


Fig. 2. Rates of fermentation of glucose by dried brewer's yeast plotted against time. Orthophosphate concentration as marked on curves.

Table 2. *Maximum rates of fermentation by dried yeasts (brewer's, Sauternes and Champagne) of* d*-fructose and* d*-glucose with varying phosphate concentration (25°)*

Type of yeast	Initial concentration of orthophosphate* (M)	Maximum rates of fermentation: With 0·049 M *d*-fructofuranose (ml. CO_2/2 min.)	Maximum rates of fermentation: With 0·252 M *d*-glucose (ml. CO_2/2 min.)	Ratio of maximum rates (f./g.)
Brewer's top yeast	0·022	1·83	1·83	1·00
	0·033	2·60	2·42 (2·38)†	1·07
	0·050	3·55	3·02 (3·02)	1·18
	0·077‡	4·28	3·12 (3·10)	1·37
	0·120‡	4·18	3·12	1·34
Sauternes yeast	0·023	2·18	2·16	1·01
	0·034	2·48	2·30	1·08
	0·051	2·84	2·48	1·15
Champagne yeast	0·027	1·94	1·88	1·03
	0·055	2·62	2·30	1·14

* Added orthophosphate only.
† The figures in brackets refer to the maximum fermentation rates obtained with 0·222 M-*d*-mannose as substrate.
‡ With this phosphate concentration 0·054 M-fructofuranose was used.

than with glucose as substrate, an observation already made by Harden & Henley (1921). The ratio of the maximum fermentation rate of fructose to that of glucose by dried brewer's yeast at equal initial orthophosphate concentration rises from 1·0 at 0·022 M-phosphate to 1·37 at 0·077 M-phosphate (Table 2). $MnCl_2$ (5×10^{-4} M) and $MgCl_2$ (1×10^{-2} M) were found to be without effect on this ratio. It may be pointed out that for a fair comparison of the respective maximum fermentation rates at equal initial phosphate concentration it is essential to shorten or to abolish by the addition of hexosediphosphate and acetaldehyde the induction period of fermentation. With dried preparations of Sauternes and Champagne yeasts the values for the ratio of the maximum fermentation rate of fructose to that of glucose are nearly the same as those obtained with dried brewer's yeast at the corresponding phosphate concentration (Table 2). Increasing the orthophosphate concentration to 0·077 M did not further affect the maximum fermentation rates by the dried wine yeasts.

Table 3. *Acid-soluble orthophosphate content of living and of dried brewer's and Sauternes yeasts*

Type of yeast	Dry moist wt.	Acid-soluble ortho-P: mg./g. moist yeast	Acid-soluble ortho-P: mg./g. dried yeast
Brewer's top yeast	0·2794	0·84	3·34
Sauternes	0·2701	0·82	3·45

From the figures in Table 3 and the density value $d_4^{18} = 1{\cdot}093$ g./ml. for our yeast it may be calculated that in living yeast cells at the onset of fermentation the orthophosphate concentration amounts to about 0·038 M (cf. Macfarlane, 1936).

The results recorded in Tables 1–3 afford evidence that the ratio

$$\frac{\text{maximum fermentation rate of fructose}}{\text{maximum fermentation rate of glucose}},$$

referred to 0·049 M-fructofuranose, 0·252 M-glucose and 0·038 M-orthophosphate concentration, is 1·02 with living brewer's yeast, 1·28 with living Champagne yeast and 1·56 with living Sauternes yeast, whereas with the dried preparations of the above yeasts this ratio becomes 1·03–1·04 (extrapolated) in each case, if the pre-existent orthophosphate content of the dried yeast is taken into account (Table 3).

The difference between living and dried Sauternes yeasts in their behaviour towards fructose and glucose is still more spectacular when invert sugar is used as substrate. Table 4 exemplifies the well-known fact that living Sauternes yeast selectively ferments fructose from invert sugar (conditions: 1 g. Sauternes yeast in 20·0 ml. total volume). This capacity of the living Sauternes yeast is lost when the cells are thoroughly dried and ground. When the fermentation of invert sugar by dried Sauternes yeast is interrupted, *d*-fructose predominates in the residual mixture of glucose and fructose, just as it does with dried brewer's yeast as the fermenting agent (Table 5).

Table 4. *Selective fermentation by living Sauternes yeast of* d*-fructose from invert sugar (in nitrogen at* 25°)

Time (hr.)	CO_2 produced (ml.)	*d*-glucose present (mg.)	*d*-fructose present (mg.)	*d*-glucose consumed (%)	*d*-fructose consumed (%)
0	0	500	500		
2	84·8	448	95	10·4	81·0

Table 5. *Analysis of fermentation of invert sugar by dried brewer's and Sauternes yeasts at* 25°

Type of yeast	Invert sugar added (mg.)	Fermentation interrupted after (min.)	Residual glucose + fructose after interruption of fermentation (mg.)	Optical rotation of the liquid (removal of the residual glucose + fructose) (deg./dm.): Before	Optical rotation of the liquid (removal of the residual glucose + fructose) (deg./dm.): After	Glucose recovered (calc.) (mg.)	Fructose recovered (calc.) (mg.)	Percentage of glucose consumed	Percentage of fructose consumed
Brewer's top yeast	1000	65	309	−0·41	+0·75	68	241	86·4	51·8
	1000	63	312	−0·52	+0·78	54	258	89·2	48·4
Sauternes yeast	1000	150	343	+0·20	+1·31	95	248	81·5	50·2
	1000	111	391	+0·08	+1·08	136	255	73·9	49·0
	1000	178	380	+0·39	+1·26	144	236	71·9	52·8

If dried Sauternes yeast is suspended in water and shaken for 15–20 min. at 25° a substance with a strong dextrorotatory power is extracted (without concomitant fermentation). On further analysis it was found that this substance is very slowly fermented by fresh baker's yeast, is soluble in 60% ethanol, does not reduce Fehling's solution, is resistant to acid hydrolysis, but finally—after 6 hr. heating at 100° with 1 N-H_2SO_4—yields a reducing substance which does not give the Seliwanoff reaction. From the neutralized hydrolysate a phenylosazone was prepared which melted at 204° and did not depress the m.p. of pure

glucose-phenylosazone. Since these properties are identical with those of trehalose, a disaccharide known to occur in many yeast strains (for literature cf. Kluijver & van Roosmalen, 1932), the substance referred to is most probably trehalose. From our dried brewer's top yeast only very small amounts of this substance could be extracted under the same conditions. The dextrorotation observed in the main assay with dried brewer's yeast after removal of the residual glucose and fructose may be due partly to trehalosemonophosphoric ester which is normally produced in the fermentation of fructose or glucose by dried yeast (Robison & Morgan, 1930). In the case of dried Sauternes yeast fermenting invert sugar the formation of this ester may contribute to the positive rotation exhibited by the suspension fluid after removal of the residual hexoses.

DISCUSSION

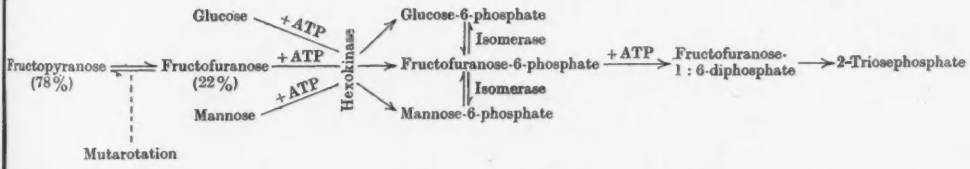
The experimental result that with dried brewer's yeast the rates of fermentation of fructofuranose and of glucose increase with rising orthophosphate concentration indicates that under the conditions prevailing the enzyme reaction:

$$\text{3-phosphoglyceraldehyde} + \text{orthophosphate} + \text{cozymase} \rightleftarrows \text{1:3-diphosphoglyceric acid} + \text{dihydrocozymase} \quad (1)$$

controls the rate. The further fact that with dried brewer's yeast the ratio

$$\frac{\text{maximum fermentation rate of fructofuranose}}{\text{maximum fermentation rate of glucose}}$$

rises from about 1·0 to 1·4 when the orthophosphate concentration is increased from 0·038 M, the level obtaining in the intact yeast cell, to 0·080 M may best be interpreted in the following manner. It is assumed that, when the rate of reaction (1) is increased beyond a certain level, the conversion of glucose-6-phosphate into fructofuranose-6-phosphate becomes the factor limiting the rate of glucose fermentation. This reaction, catalyzed by hexose phosphate isomerase, is involved in the fermentation of glucose, but unessential for the fermentation of fructofuranose (cf. scheme). This interpretation conforms very well to the observation made that the maximum fermentation rates of *d*-glucose and of *d*-mannose by dried brewer's yeast coincide for all orthophosphate concentrations tested (cf. Table 2).



The main result of the present investigation is that the selective fermentation of fructose from invert sugar exhibited by the intact Sauternes yeast cell is reversed when dried preparations of the same yeast are used, thus aligning the behaviour of dried Sauternes yeast towards invert sugar with that of living and dried brewer's yeasts. This finding suggests that the membrane of the intact Sauternes yeast cell is more permeable for fructose than it is for glucose and that the selective fermentation of fructose by the living cell results from the preferential permeability of its membrane for fructose and from the higher affinity of fructofuranose for hexokinase.

Our results with dried Sauternes yeast are, apparently, contradictory to those obtained by Fernbach, Schoen & Mori (1928). An examination of the experimental procedure adopted by these authors reveals, however, that no allowance was made for the dextrorotation due to the presence of optically active substances extracted from and produced by the dried Sauternes yeast during fermentation. The French authors investigated four different strains (brewer's bottom yeast, *Saccharomyces exiguus*, *Schizosaccharomyces Pombe*, Sauternes yeast) as dried preparations polarimetrically in respect of their behaviour towards an aldose-ketose mixture (prepared by treating *d*-glucose with 0·8 % $NaHCO_3$ at 40°); the last three were similar in that dextrorotation was observed in the liquid at the end of the experiment, though the intact cells of *S. exiguus* and *Sch. Pombe* produced a reverse effect. Only brewer's bottom yeast, which is known to be free of trehalose (Müntz, 1876; Tanret, 1931), caused laevorotation whether it was used in the living or the dried state.

In this connexion another source of error may be mentioned. The membrane of the Sauternes yeast cell does not yield easily to destruction. If the freeze-dried yeast is ground for only one minute the resulting powder is much less active towards glucose than the one thoroughly ground. Thus, in one experiment, carried out under the same conditions as those recorded in Table 5 but with a Sauternes yeast which had been dried and then very briefly ground, 73·0 % of the fructose and only 45·2 % of the glucose added were fermented. This property of the cell wall of Sauternes yeast may account for the fact that the ratio

$$\frac{\text{percentage of glucose consumed}}{\text{percentage of fructose consumed}}$$

was usually found to be a little lower with dried Sauternes than with dried brewer's yeast (Table 5).

The evidence presented in this paper indicates that in the fermentation of invert sugar by dried preparations of yeasts (brewer's, Sauternes) the relative rates at which *d*-glucose and *d*-fructose disappear from the suspension are determined in each case only by the relative concentrations of glucose and of fructofuranose in the suspension and by their relative affinities for hexokinase. With intact cells the ratio of the relative rates at which the two fermentable sugars pass through the cell wall is an additional factor controlling the relative rates of glucose and of fructose consumption. Thus, differences in the structure of the cell rather than differences in the enzyme pattern (Duclaux, 1900; Fernbach *et al.* 1928; Sobotka & Reiner, 1930*b*; Hopkins, 1931; Gottschalk, 1944) are responsible for the varied reactions of the living yeast cells towards *d*-glucose and *d*-fructose.

SUMMARY

1. The normal response of living yeast cells to invert sugar is the preferential fermentation of *d*-glucose; it results from the unequal distribution of the hexokinase between *d*-glucose and *d*-fructofuranose, the higher concentration of the aldose outweighing the greater affinity of fructofuranose for the common enzyme.

2. The capacity of Sauternes yeast selectively to ferment fructose from invert sugar is confined to the intact cell. When invert sugar is incompletely fermented by freeze-dried and thoroughly ground Sauternes yeast, *d*-fructose predominates in the residual mixture of *d*-glucose and *d*-fructose, just as it does in fermentation with living and dried brewer's yeasts. This observation suggests that the selective fermentation of fructose by the intact Sauternes yeast cell is due rather to a preferential permeability of its membrane for fructose than to an enzymatic equipment different from that of the other yeasts.

I wish to thank Dr A. W. Turner, Council for Scientific and Industrial Research, Melbourne, and Miss F. E. Williams for their ready and valuable help in preparing the pure cultures used in the experiments.

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